*Minute-taker's note: In Q&A sections, the questions/comments are **bolded** and the answers are indented dashed bullet points beneath.

WERA-20 Meeting
July 6, 2015
Day 1

8:00 – Mingling, networking

8:30 – Greeting: Gary Kinard, USDA-ARS Chair of 2015 WERA-20 Meeting

Charles Onwulata – ARS initiatives: National Germplasm resources laboratory and pathogens, provide tools and science.

Open data to retrain scientists and bring them up to speed
- Virtual Corps of bioinformatics
- Science DMZ – using lines to enable open data to remain high speed while in high use
- Cloud system of information storage
- Deliver timely, useful, plant resources and to make use of the knowledge from the group to deliver all the products

Ellen Harris, Director of BARC – Welcome.

Jim Moyer unable to come, Hanu Pappu representing, Administrative Project for WERA

Gary Kinard – Housekeeping
15-20 additional people attending Monday and Tuesday for NGS presentations

8:43

Dr. Mark Nakhla, Director of CPHST, USDA-APHIS, Beltsville

Review of NGS Tool and Science and Technology
- Risks, screening, indexing, certification programs, etc. of NGS

Today: Identify matters of importance in the factors and applications of NGS
The 70s-80s was ELISA, 90s was PCR, Today: NGS
Sequencing platforms improving (faster, cheaper, and more efficient) and hoping to bring NGS to every lab
Want to control plant diseases at national and international level

8:48

Sarah Trujillo, Moderator

The purpose of this meeting is to bring together the scientific and regulatory community to engage in the start of a conversation around NGS and the use of this technology to find

pathogens and/or certification purposes. The group will review the current science around NGS and gain a better understanding of each other's need to start identifying a collaborative process for moving forward with the use of this technology.

Looking at ways of moving forward together as a community

8:50

Dr. Maher Al Rwahnih, Project Scientist, Foundation Plant Sciences, Davis, California
Next Generation Sequencing (NGS) Technologies & Current Applications

Sanger Sequencing ruled the world the last 30 years

- Issues: sequencing costs remained high
- Speed to generate data remained low
- Involves cloning procedures

NGS Sequencing Revolution

- Millions of short sequences generated for a given DNA sample
- Comprehensive picture of the entire microbial profile
- Can sequence 900 GB of DNA per run, enough to fully sequence complete genomes

Second Generation Sequencers

- Limitations: difficulty to read through repetitive or homoploymer regions (GS FLX Titanium XL + Systems), relatively short reads (Illumina HiSeq X), and short reads (SOLid)

Third generation sequencer: Benefits of the 'Sequencing Wars'

- The competition is making sequencing faster and cheaper
- They offer a set of new advantages:
- The MiniION: in alpha testing: a membrane technology that promises a quantum advance in read length, and similarly impressive decrease in cost
- Bench top sequencers
    - Limitations: Relatively short reads (Illumina NexSeq), short reads (Ion Proton Sequencer)

Which platform is best?

Next Generation Sequencing Cost

- 5K for 6-8M reads
- <500, 60-70M reads

Why NGS Tech?

NGS Current Applications

- "Plant Virus Metagenomics: Advances in Virus Discovery"
- Grapevine:
    - Study diseases of unknown etiology
    - Metagenomic virome analysis
    - Re-sequencing of known viruses
    - Host-pathogen interactions
    - dsRNAs were used to synthesis cDNA libraries
    - Vein-clearing infection from the Midwest region of the US
    - GRBaV, DNA virus
    - Graft incompatibility – discovery of a novel virus satellite
    - Discovery of the first reovirus in grapevine

  - Novel viroid was discovered in infected Pinot Noir
  - Virome of an entire vineyard
    - 45 grapevines
    - Mycovirome of a plant – 26 putative fungal virus
    - Used for re-sequencing
  - Re-sequencing of known viruses: a new strain of GLRaV-3
  - Host-pathogen interactions – high-throughput sequence analysis of small RNAs in grapevine

Limits to NGS based characterizations
- Bioinformatic treatments cannot prove pathological causality
- Detection of a given pathogen sequence does not mean that the given pathogen is responsible for the disease
- Koch's postulates cannot be satisfied using only NGS-based data

Discovery of some insignificant background viruses
- Cryptic viruses
- Latent viruses
- Asymptomatic viruses

Our main goal is to single out the viruses with *high economic impact*

Mixed infection
- The most pathogenic viruses in a mixed infection cannot be distinguished from the rest by NGS data

Identifying the causal agent – correlative association is not enough
- Finding the putative pathogen in a symptomless plant
- Finding symptomatic plants free of the putative pathogen
- E.G. found GSyV-1 in both symptomatic and healthy-looking vines

The problem of unidentified reads
- We cannot know if there are any hidden pathogens in the "unknown reads category"

Possible solutions?
- New methods that do not depend on BLAST homology searches:
  - Separate sequences into clusters based on physical characteristics of the sequences

9:05

Dr. Ioannis E. Tzanetakis, Associates Professor, Plant Virology, University of Arkansas,
Presented by Maher Al Rwahnih
"Sample Preparation"

Cleaning of work area – basic but very important
- Work areas are cleaned with germicidal wipes, RNAse away spray and absorbent pads are laid down
- Pipettes are cleaned with germicidal wipes, RNAse away spray and UVed for 30 minutes on each side
- Magnetic stand, tube and plate racks are cleaned with germicidal wipes

Preparation of NGS libraries
- First method: using TNA as a template
- Extraction of total nucleic acid (TNA) using TNA Extraction
  - 1g of petioles or bark scraping is pulverized using liquid N

    - 10ml of extraction buffer is added to the ground tissue
- Quantification and Quality Control
    - Dilutions needed for quantification
    - Quantify RNA
    - Check quality of library with Bio-Analyzer HS DNA assay
- Library Preparation Outline
    - Illumina TruSeq Stranded Total RNA Ribo-Zero Plant Kit
    - Water control included
- Second method: using dsRNA as a template
- WGA Protocol – denaturing step
    - cDNA synthesized set up in a biosafety cabinet
    - Amplified with GenomePLex
- Sonication of DNA
- Quantify DNA after Sonication with Qubit BR DNA assay
    - Check quality by running Bio-Analyzer
- 100ng of fragmented DNA → Repair ends and size select → Adenylates 3' Ends → Ligate Indexed Adaptors →
- Quality Control for Library – **overlay traces** of DNA before and after ligation to verify the adaptors ligated correctly
- Third Method: Using siRNA as a template
    - Commercial kits like mirPremier microRNA isolation kit
    - In-house protocols using Trizol
    - Library prep using TruSeq Small RNA kit

Automation of NGS sample prep for large scale application
- Need to:
    - Develop and validate new sample preparation protocols
    - Improve efficiency
    - Decrease costs
    - Increase throughput
    - Decrease sample tracking errors
    - Increase productivity

Sequencing Facility Processing
- QC libraries, using Bio-Analyzer and qPCR
- Libraries pooled in equal molar ratios
- Loaded in flow cell

9:18

Dr. Ioannis E. Tzanetakis
"Virfind: Bioinformatics made easy"

Open access – everyone can use it

Building the library: nucleic acids
- Using double stranded RNA, siRNA, rRna and DNA (-) nucleic acids
- siRNA time consuming

cDNA Synthesis and Sequencing
- Degenerate oligonucleotide primed RT-PCR

VIRFIND computer node

- Dell PowerEdge
- 192 AMD processors
- 1.5 Tb RAM memory
- Remote access
- Housed at Arkansas High Performance Computing Facility
- Local tools: NCBI Blast, Fastx Toolkit, etc.

The pipeline

- Input files collapsed and put into a faster format
- Take out bar codes
- Run Multiplex
- Take sequences and align and test them against genome-reference sequences found in database – takes out stuff we don't care about
- Assembly with host genome afterward –
- Detection: blast to GenBank at database to find known viruses
- Finding new viruses
    - VIRFIND: beta-testing – outside users
- Plant hosts: brassica, berries, etc.
- Animal hosts: honeybee, human
- All viruses identified with other methods also present in VirFind results
- New viruses discovered, undetected when using the favored methods
- Low vs. high expectation values on virfind.org site – changes speed of site
  An example in Ribes

9:35

Dr. Maher Al Rwahnih

Bioinformatics Tools: CLC Genomic Workbench Demo

1. Importing data
2. Trimming Sequences
a. Trim Settings
3. De Novo Assembling
4. Extracting Contig
a. Blast Search – slow process, not efficient to do with CLC
b. Export to VirFind for higher efficiency
c. KLAST Search – similar to VirFind to look for viruses using specific virus genomes
i. Different options: standalone license or plugin
5. Batch Edit
a. KLAST – sequence bank comparison tool
   Expect false blast hits! Further verification

9:43 – Break

10:15

Dr. Ioannis Tzanetakis

"Verification: You now have a virus, so what…?"

Dealing with regulations in latent viruses.
Bioinformatics: you cannot believe everything you see…
- Always verify the results with a second approach
- Start from scratch – don't use the same template
- ELISA (known), PCR/Hybridization (unknown)
- Contamination happens
- Mycoviruses are always present in field samples
- Investigate: a lot of dsDNA viruses have similar motifs to transposons
  Example of Blackberry Yellow Vein Disease (BYVD): one disease, many viruses
- THE disease in SE USA
- Symptoms vary
- Caused by multiple viruses – asymptomatic in single infections
- Virus distribution
    - Collected a lot of samples to see whether this virus is present in a significant amount of blackberries
    - Found virus in 60%

Badnavirus –
- Virus integrated in host genome – may lose their capacity to cause disease
- Integration of virus in genes = can't get it out of the host plant → weak link in getting clean plants
- Indicator plants
    - Have to know if the virus is active or inactive – that's what makes you care about the discovery of virus presence. RT PCR to determine whether or not it's active
- Population structure: BYVaV – Multistate sample collection – 35 isolates
    - Overlap of two genes = good for primers to help identify virus

Virus distribution take 2:
Bioinformatics and the discovery of asymptomatic viruses: The BYVaV/BVY story
- BVY did not cause symptoms in single infections but together with BYVaV they cause BYVD
- In mixed infections, BVY knock down concentration of BYVaV to about.1% compared to titer in single infections
- In mixed infections, they can cause death of fruiting canes
- Latent viruses are latent viruses, but you may miss a lot of important information if you don't do the biology

In a nutshell: Bioinformatics vs. Biology
- A new virus does not constitute the apocalypse, even if it is widespread
- Blueberry Latent Virus:
    - 10-year study – the virus is present in 52% of all Germplasm
    - 100% seed transmission
    - A true cryptic virus, no symptoms in single or mixed infections

No decision can be made on the importance of a virus without biology

10:42

Dr. Maher Al Rwahnih

"Comparison of NGS vs. Biological Indexing for the Optimal Detection of Viral Pathogens in Grapevine"

New Grape Introduction (foreign import, domestic selection, new variant) → Required disease testing (lab testing PCR, ELISA, bioassays for woody and herbaceous) → Disease Elimination (tissue culture) **All tests negative** → Provisional Foundation Vines, Professional Identification, ID verification, Registered Foundation Vines → Nurseries and Growers. Takes 2-6 years

Standard detection methods for ID of viruses and virus-like pathogens (ELISA, PCR)

Bio assays using herbaceous hosts

Woody indexing (chip-bud inoculation)

Biological indexing

- Advantages:
    - Broad spectrum:
    - Able to detect the majority of agronomically significant pathogens
    - Able to detect previously unknown agents
- Drawbacks:
    - Does not detect all pathogenic viruses
    - Time consuming (2-3 years)
    - High cost in greenhouse and field space and labor
    - Cannot specifically identify pathogenic viruses – further steps required
- Reliability: variable, conditional on
    - Success of bud-take
    - Transmission of the virus from the bud to the indicator vine
    - Effects of climatic conditions on symptom expression
    - Virus species and strain

Can NGS replace the field indexing requirement?

- Need to scientifically demonstrate the advantages of NGS over Bio Indexing – side by side comparison
- Pathogens to represent regulated viruses
- 54 different grapevines (including healthy ones) and compared
    - NGS shows more viruses present than simply using bio indexing – more comprehensive picture of viruses
- Superior Detection Sensitivity
    - NGS identified 21 plant virus species and strains were detected, 2 viroids and a satellite RNA were also detected, as was a list of myco/cryptic viruses
    - NGS identified grapevine leafroll viruses to the species level where the CF indicator only identified generic leafroll disease
    - NGS detected infections by GRVFV, GSyV1
- **Comparative sensitivity – bio assays failed to detected 5 regulated viruses**

Time Requirements:

- 2-3 years for bioassays
- No more than a MONTH for NGS analysis

Preliminary Findings:

- NGS analysis was found to be superior to the standard bioassay in
    - Detection of viruses of agronomic significance
    - Its comprehensiveness
    - The speed of its analysis

  - o The discovery of novel, uncharacterized viruses

NGS as a replacement for the biological index assay

- **When NGS use is accepted for grapevine certification and registration in place of the current industry standard, growers will be able to start propagative increase and virus elimination programs with most new accessions years earlier than they can now.**
- Improved testing protocol: Release time using NGS: 2-4 MONTHS

11:00

Q&A

Viruses found in NGS would have sent the accessions back to testing

6-7M reads for 1 virus

Have you tested to see if the RT sampling is important?

- We had some experience with the RT, and found those viruses can be detected several times. . . sampling at different times of the year to find different viruses. Do comparison with real time and NGS to see if we're missing anything
- It really matters what time of year you do the biological index, but need 20 sticks doing biological indexing. If you do NGS you can in good conscious test 5 sticks and find all the viruses in regulation you need to look out for

Uneven distribution of NGS? Provisional release after the first NGS and full release after second NGS?

- Grapevine: uneven distribution not an issue.
- Provisional: propose provisional release after first round - instead of waiting we can send it to customers immediately

Running double stranded RNA

Find 85% of whole genome, but haven't proven it's actually infections.

- Only looking at regulated virus. Not making virus-free
- Not looking at background virus
- Focus on regulated viruses and make sure our plant stock is free from them
- Any new virus, need to do a 10-year study (esp. in woody plants)

At what point do you think NGS is contaminating something?

- All your reads are in one area – one possibility
- A viroid on a presumed-healthy sample – have to retrace steps and see if there was a mistake
- Repeat the samples one more time in NGS
- Start from scratch – take leaves from every part of a plant, extract, and take a portion of that
- NGS with 3 rounds with 3 hits, can't test with PCR or ELISA – can't do anything about that. It's a 99.9% accuracy testing

Advantage of qPCR if we only deal with a few viruses?

- NGS can cover all the strains of any virus. qPCR even if you try hard to cover the strains, you're dealing with a biology agent that can make a mistake.
- Tool to discover all the viruses, but not all the viruses it finds are "novel"

Do you envision you'll be able to set threshold limits to consider virus-positive plants?

- Future research: more samples – this amount of leads – artifact to compose a threshold
- What we need to look at is: is it really a new virus?
- Novel viruses 2009-2015.
- "Grapevine Red Blotch-Associated Virus, and emerging Threat to the Grapevine Industry"
- Molecular variability
- Is it a novel disease, or caused by an invasive introduction?
    - UC Davis Center for Plant Diversity/Herbarium. Houses 300K dried samples
    - Sample collection: 56 grapevine specimens collected
        - Specimens were harvested and pressed between 1937-1959
        - Pieces of foil containing each sample were placed in individual Ziplock bags to prevent cross contamination
        - Tested for GRBaV using both conventional and Quantitative PCR assays. One sample turned out to be positive
    - Genomic analysis – obtain the full genome sequence of GRBaV herbarium
        - Confirmation by NGS
        - NGS generated about 88M Illumina reads
        - More than 92K reads mapped specifically against the GRBaV genome
    - Blast Results – the full genome sequence of this herbariums isolate shared 92-99% nucleotide identity with other GenBank isolates.
        - The virus was a member of a mixed infection with GLRaV-2 and GVB
        - **Not a novel virus, been around 75 years**

1940 specimen was imported from France?

- History of the Early Burgundy was from France
- "Early Burgundy" is no longer used, but the term is a California term for grapevines imported from France in the 1880s before there was a quarantine system in place that could be used for making Burgundy wines
- Internationally, colleagues have been slow to test material in their countries

What material did you use and how did it compare to the RNA?

- Used RNeasy and the quality was fantastic
- Amount of data gathered

Have you used Trizol?

- Trizol doesn't work in grapevine. I bet my career on it.

Old “new” diseases only found when the new technology came out. We don’t see any symptoms, the nicest looking peach has the most diseases. We cannot identify a “sick” plant due to the viroids

- Sometimes they are asymptomatic

Do you test plants in the US only?

- It’s also in Canada
- 5% of grapevines show presence of Peach Latent Viroid in Ontario and Niagara region

Does it concern you that as newer techniques are available we are moving away from things like virus purification? Now we have NGS, will we not do ELISA in the future? Are we going to modify the existing hypothesis on NGS?

- We have to use a second method, even sometimes a 3rd method. NGS by itself is a good tool but we don’t want to be misled. PCR, even if you can look at the virus particle, it’s hard in woody crops but if you can see it it’s a good thing to do.

Using NGS for post-therapy?

- Yes

Taking the cutting, push new shoots and put into therapy. We do that in CPHST, tell me what you’re doing when we see 5 diseases in the greenhouse, but all our efforts to identify pathogens with NGS?

- 99.9% accurate. Bioinformatics is based in the previous knowledge.
- Which method would you prefer? We need to have the perfect test: we are all going lose. Nothing is perfect.
- It may be time to issue new permits for NGS for practitioners crop by crop. But could we have a meeting on a few crops where we could have an experimental permit to use NGS the way we’ve been talking about it

Questions for Ioannis

We have the capacity for processing, but we don’t have capacity for access, is there a way to make the script available as a download tool?

- We can do that, that is not a problem, but you will be missing the updates. It was published in December, and though it’s only been 6 months we’ve made some good improvements already

12:00 Lunch

1:00

Dr. Maher Al Rwahnih
Cost Analysis

Instruments for extraction and QC: Instruments:

- Agilent Bio-Analyzer 2100: $21K
- Life Technologies Qubit 3.0: $2K

Bench Top Sequencers

- Illumina MiSeq: 125K, 25M reads
- Illumina NexSeq: 225K, 130-400M
- Life Technologies Ion Proton: 225K, 60-80M
- 454 GS Junior system: 100K, 1M reads

Bioinformatics resources

- Online open-source software
- University bioinformatics Cores of labs
- Bioinformatics companies: CLC bio, DNA Star, Geneious, Korilog
- User friendly bioinformatics tools for data analysis:
    - Genome browsing and annotation
    - Alignment of sequence reads to a reference
    - SNPs detection
    - *De novo* assembly
    - Sequence similarity search (BLAST)
- Windows based Bioinformatics
    - CLC Genomics Workbench license:5K
- Linux Based Bioinformatics
    - Advantages: run analysis automatically using scripts
        - Built in Unix tools to analyze data
        - Many new assembly programs implemented in Unix
    - Disadvantages:
        - Need familiarity with the command line
        - Hard to get started
- Linux Cluster
    - Network of fast computers connected together
    - CPU: 4 Intel Xeon E5 8-Core processors RAM: 512 GB: 15.5K
- Library Prep Kits
    - Price per sample ranges from $8.00-5K
- In-house cost of Library Prep
    - $250 using Illumina kit
- Out-sourcing library prep and sequencing range from $60.00-2.4K at UC Davis, and $5.00-4.5K in industry

With NGS Testing option

- Cost: $900 per selection. Total testing time is 1-2 months
- Main advantage: detects all known and unknown viruses (and strains)

Conventional virus testing costs: PCR + ELISA + herbaceous host indexing, + woody host indexing = $1900 per sample

Library Prep: in your experience, which is the best?

- Tested with Kappa, Illumina, and Commercial kit and they react basically the same

1:12

Dr. Ruhui Li

"RNA-seq Platform for Virus Diagnosis in Fruit Trees: Ready for Plant Germplasm Quarantine?"

Bioassay: long process – 2 years
Problems: unknown pathogens, multiple detection assays, large space, 2-3 years
Application of NGS in virology

- Identification, characterization, population structure and diagnosis of pathogens
- Different starting materials
- Different platforms
- Different bioinformatics tools
- Reference genomes

RNAseq for virus identification

- Total RNAs → Illumina Hiseq 2000 → Bioinformatic analysis → Virus verification
- RNA taken from leaf samples, healthy and diseased

RNA HiSeq Results

- Mapped against apple genomic, chloroplast and mitochondrial contigs

Pathogen identification

- *De novo* assembly

Positive Control Z3005 – has ACLSV and ASPV had 81% identity in ACLSV
Apple indicator "Spy" by Q47930 – originally thought to be clean, but after being put onto Spy, showed signs of virus. Used PCR to detect 3 viruses on it: ACLSV, ASPV, and ApMV-like
Z3034: Source: accession from Uruguay.

- Symptoms: stem swollen on original tree, but not on indicator plants
- Transmission: grafting and . . .?
- Host range: some apple cultivars
- Causal agents: ACLSV, ASGV, ASPV, and . . .? Latent
- Looks to be a new combination of viruses
- Found new virus, with two genetic variants of ASGV, ASLSV: 5 genetic variants: ASPV: >18.
    - New virus in high concentration
- Verification: virus detection, graft transmissible

Conclusions: Illumina RNA-seq and bioinformatics analysis using CLC bio are reliable to detect pathogens with RNA genome

- Large contigs generated from RNA-seq data allow the identification of not only different pathogens but also different variants of a virus
- Sample preparation is easy
- Further study necessary to determine its potential for detection of pathogen with DNA genome

1:30

Dr. Mike Rott, Canadian Food Inspection Agency
"NGS: The Canadian perspective: NGS for the Detection of Tree Fruit and Grapevine Viruses with the Goal of Bringing this Method into our Post-Quarantine Diagnostic Lab for Routine Use"

- Roles and Responsibilities:
- Activities governed under the Plant Protection Act and associated regulations
- National post entry quarantine facility to prevent the introduction and spread of plant virus and virus-like diseases

- Virus elimination
- Tissue culture
- Virus-free repository
- Southern Vancouver Island

Test Samples come from foreign non-certified sources

- Audit samples from approved certified sources
- National surveys

Pros of Virus testing

- Works well
- Detects most characterized viruses
- Detects new viruses

Cons

- Slow

5200 individual tests are performed on the 30 samples over 1-2 years (up to 3 for tree fruits)

Promise of NGS

- Samples processed in days/weeks instead of months/years
- 1/10th the cost
- As sensitive as PCR
- More accurate
- Greater sensitivity
- NGS testing: 90 tests

Workflow

- Bark cambium 1-5g, double strand dsRNA, total RNA, smRNA
- Composites
- Nucleic acid extraction
    - dsRNA samples longer
    - Sample cross contamination
- Extraction QA and Quantification
    - Fluorescence
    - Bioanalyzer
    - Gel Electrophoresis
    - PCR
        - Used French Bean: Black Turtle Soup to give consistent level of target for consistent PCR results
        - Has consistent titer, seed transmitted, easier to grow
- Library Prep
- Sequencing: in-house vs. outsourcing → 454 vs. Ion Torrent vs. Illumina vs. Solid → MiSeq vs. NextSeq vs. HiSeq → 75 base kit (→detection→ 24 samples/run, 16M reads/sample, $130/sample total cost not counting labor costs) OR 150 base kit → genome assemblies
- Data analysis
    - Each sample = several GB of data → TB of data
    - What is the goal?
        - Detect/quantify known virus species?
        - Distinguish between virus species variants?
        - Detect new virus species?

        - Assemble viral genomes?
        - Different approach required
    - Read Mapping: Positive determined based on
        - Number of percent reads matching target
        - Coverage
        - Depth of coverage
        - Weight: proportion of reads mapping to sequence
        - Best hit: proportion of reads with highest mapping to sequence
        - Internal control

Detecting unknown viruses workflow
- Subtract reads mapping to database of viral sequences
- Subtract reads mapping to database of host genome
- *De novo* assembly of remaining reads into contigs
- Scan contigs for ORFs
- Translate ORFs into protein sequences
- Screen protein sequences using Vfam
- Identify sequences with significant score for further analysis

Virtool: in-house developed workflow for virus detection
- Based on Pathoscope
- Easy to navigate GUI
- Design build to comply with ISO-17025
- Data QA: Integrity, access control, retrieval and confidentiality/security of electronic data, documents and records.

Determine visually if primers will work – currently working to develop a means of quicker primer validation

Sample Analysis Summary
- Processed over 600 samples to date
- Assembled over 2000 viral genomes representing over 100 species
- Discovered more than 20 new virus species
- Directly compared over 125 samples: NGS vs Conventional Methods
    - NGS is 2-3 times more accurate

NGS 2015
- Starting to use NGS within Diagnostics Lab
    - All audit, non-certified and ad hoc samples are to be processed using NGS and conventional methods for direct comparison
- Collaborating with CFIA animal and food virus labs

NGS 2016
- Interest from grapevine industry – bringing NGS into greater use

2:00

Q&A

For Rhui: 5 virus strains of ASPV
- All 3 match 100% to the NGS-generated genome.
- Found 5 different variants

- Apple Green Crinkle Disease – conclusions lead to certain variants of ASPV because they were unable to detect other variants
- The lower the n-number, the lower flexibility you have to rule other things out
- Almost invariably find multiple variants of ASPV

How do you know you're detecting variants or a mutation?

- You see both, but you can clean it out to see different variants and also variations
- High overlap and similarity
- Variation is across the genome in some species

NGS is more sensitive than PCR – should you use only one or both?

- You can detect from NGS to PCR, but not always vice versa

When you send the sequence out and it comes back months later, how does the turnaround time affect sequencing?

- I can do 24 extractions, set up the libraries and sequencing on Monday and I'll have all my results on the next Monday, so 1 week

You have several steps: do we need to set quality control for each step?

- We have our internal control for extraction, we can still get cross contamination, but we do a control and negative control. When you're doing your libraries you do the same thing. Hopefully when we get the neo-prep, I'm hoping that'll help better. It's a very tricky thing, you're doing regular diagnosis that's screaming hot and another that's next to nothing, and you have both of them together it's hard to compare. That could easily be contamination if you're not careful.

Do you have any amount of data you need as a minimum? Is there any thought as to the minimum amount of data?

- We have found a new virus from a single read. You can have contamination, but you can find a new virus from only one read
- You don't want to risk missing anything.

What are the possibilities of finding new pathogens?

- We have the first version of a type-line that can find nematodes and pathogens

You said you discovered 10 new viruses, are they comparatively new?

- They're new, a mix of geminiviruses, flexiviruses, etc. You just find them
- I didn't find any new family, but I haven't had time to look through the new ones I've found
- Keep in mind we'll find a whole lot of stuff. It's going to create a situation that we'll find a whole new lot of information we haven't seen before. I vote on a policy that there be no policy until we have more data

We don't know what these "new" viruses do, and putting a policy on it might make us have to wait 8-10 years, and that's too long. Agree with Deborah to have no policy until we know more about these viruses and if they cause harmful diseases. How do you "know" what these viruses do?

When you're dealing with material that's originating in the country, you can be far more tolerant to things you find than you do to material coming from outside the country. Especially into a vegetatively propagated crop, it will have been propagated millions of times and distributed around the country. Viruses of currently little importance have been found to increase to significant disease proportion after other things have been cleaned up. Have to be careful what you label as an unimportant disease.

Raise the question about timing: CVA found 18 years ago, and just this year we're understanding the distribution. What do you do in that introduction? How do we manage with those viruses in the meantime of 6-7 years for horticultural info? So much international distribution, they don't want these viruses going to their country, but if they find viruses in our exports that looks bad on us.

We should not have a rule, we should be open. This is not one rule fits every situation

Possibility of viruses being beneficial?

- One citrus tree is 145 years old, still producing fruit, and is positive for a viroid. This particular viroid enhances fruit quality though, and does not induce any diseases of economic importance.
- Mycoviruses and viroids in grapevines make the fruit consistent for wine production
- Asian Prunus Virus was unknowingly introduced in interstems that were being used to delay bloom and avoid early season killing frosts.

Multiple genomes/multiple virus variants: it's easy to understand how you get different virus variants introduced. But when you have high numbers of variants, I wonder how many of those are functional genomes, and how many are non-functional genomes? Ruhui, you said you could identify 3 variants, but for the other 3 variants you could not verify, could your colleagues verify the genome?

- At this point, I don't know. Why I got 3 is because I already sequenced 5. I tried to design primers to differentiate between the different genomes.
- There's a French paper coming out that shows how two viruses compete and they do not infect the same cells.

3:15-4:15

Group Discussion

Group A: In relation to finding new pathogens
Group B: In relation to certification

Questions:

1. To what extent are we ready as a scientific community to implement NGS as the main tool for screening Germplasm? If we are not ready, what are the gaps that still exist and need to be closed in order to be ready?
2. As a scientific community, do we know what we need from policy to move forward in NGS?
3. What is it that we think we need from policy?

Group A:

1. What should we look for in the data to believe it/trust it?
    a. What are the minimum requirements to identify it as a main tool?
    b. Peer review is not the gold standard anymore
    c. Guidelines from experts on what to look for
    d. Are there set parameters for the scientific community? Need those parameters. Second confirmation visit
    e. MIQE (minimum information for quantitative)
    f. First needs to be a valid report of a new organism
    g. What do refer to them as a group if not referring or known as a virus?
    h. Virus signature “looks like a virus”
    i. Survey – widespread of isolated incident?
    j. Overlap of science and politics
    k. EPPO/NAPPO involvement
    l. Timelag/time considerations
2. –
3. What do we need from policy?
    a. More biological information
    b. Timeline for implementation (include a grandfather clause)
    c. Incentive (carrot) for industry

Group B:

G1:

1. Import permits
2. Clean Plant Production – domestic
3. Export
4. Re-certification of G1-G4 back to G1

NGS will HAVE to be incorporated into clean stock (G1) production

- Enough validation yet?
    - Comparison with current methods against many possible controls
    - Understanding performance characteristics (precision, accuracy, etc.)
- Optimization of NGS across crops beyond virus
- Are there “certification” scenarios where NGS can be incorporated sooner than can be done for import/export?

We need to <u>lead</u> the move to incorporate NGS

What do we need from policy?

- Description of what is appropriate validation (and cost to do it)
- List of who POLICY is and path to acceptance
    - NAPPO?
    - USDA-PPQ-PHP-RPM Plants
    - Plants for Planting
    - Import policy
- “Certification” ramifications when new sequences are found.

- Path to determine whether to add to regulatory list
- Protocol to flush out newly discovered virus from existing G1
- Policy on permits for interstate movement of stock known to have newly discovered virus?

New Pathogens

- NGS is **ready** for discovery but needs biology follow-up (not only tool, ever)
- Is it a pathogen or a friendly sequence? Who sets the criteria?

In terms of discovery, do we still need comparison testing? Policy comes from publishing groups

- What is the process for regulatory status evaluation and determination for a newly discovered virus?
- - IPPC-NAPPO-US
- Is it only a problem when found in asymptomatic plants. . . would only test asymptomatic plant in a certification setting?

WERA-20 Meeting
July 7, 2015
Day 2

8:25

Osama El-Lissy, Deputy Administrator, USDA-APHIS-PPQ

In APHIS PPQ we have two goals:

1. Safeguard American agriculture
    a. Safety mitigations for specific plants
2. Facilitate safe trade
    a. Customs inspections to ensure commodities are in compliance with our standards of quality to minimize exposure to contamination
    b. Exporting commodities globally – it's very important to our society and economy (1.2M jobs in the US)

How effective are we?

- Being an effective safeguarding force, we save a lot of American money – therefore we can't be stingy in investing in the technology and skills needed to be effective.

Regulatory policies and science

- **Science has to drive policy**
- Bringing scientists and policy-makers together – where strong individuals come together, the outcome will surely be very great
- Risks associated with new technology
- You have our complete support as you continue to research

8:40

Erich Rudyj, Coordinator – National Clean Plant Network (NCPN)
"Regulatory Interface"

Purpose: NGS Technologies

- Bring scientists together with regulators
- Discuss the science
- Ascertain its interface with the regulatory community
- Being mindful of the impacts on industry

Objectives:

- Ascertain NGS scientific questions and issues to be posed for discussion in a regulatory context
- Get introduced to some of the regulatory science issues and instruments germane to NGS issues
- Ascertain 'opportunities' and 'gaps' for further discussion
- Engage in discussion – the only way we can learn is by talking to each other
- Propose action items for follow up

"We welcome and encourage a robust and engaged discussion about the power, potential, and risks of this new technology as we work together to develop a roadmap for the use of this technology in regulatory programs" – paraphrased from El-Lissy

Approach Park 1 – Several Brief Stimulating Presentations

- NGS Issues of Importance – Scientific Issues to Stimulate Discussion
    - Question and Issues – matters surrounding NGS technologies of relevance now to scientists and regulators
    - State of the Science – NGS and diagnostics in current practice
- A Possible Interface in the Discovery to Regulatory Process
    - The New Pest Advisory Group Process
- Regulatory Overview
    - Procedures/practices/policies that might need to be established for NGS among scientists and regulators

Approach Part 2 – A panel of Experts for Discussion

- Panelist Talents:
    - NGS scientists and process adherents
    - Diagnostic labs and quarantine center directors
    - Federal regulatory officials
    - International phytosanitary issues managers
    - Regulatory pest/pathogen identification and process managers

Approach Part 3 – Round Table Discussions

Anticipated Meeting Outcomes

- Identify the needs of the scientific community in relation to NGS
- Identify the needs of the regulatory community in relation to NGS
- Discuss impact of science on industry and regulatory community
- Identify matters of importance regarding the interface between the science of NGS application and regulatory matters
- Start formation of a process for moving forwarding with the conversation and linking the two communities

Science always informs policies and the policy-makers in their decision-making process

8:50

Deborah Golino, Director, Foundation Plant Sciences, University of California, Davis, CA

"Questions/Issues – Matters Surrounding NGS Technologies of Relevance now to Researchers, and Regulators"

The need of service work in the continuum.

Foundation Plant Services began as cutting-edge research in the 1950s.

- Mission to produce test, maintain, and distribute elite disease-tested plant propagation material; provides plant importation and quarantine services, virus testing and elimination; coordinates release of UC patented horticultural varieties; links researchers, nurseries, and producers.

Crop Programs at FPS: grape, strawberries, fruit tree, nut tree, rose, sweet potato, pistachio, and olive

CA Registration and Certification Programs to certify Prunus fruit and nut trees, strawberries, and grapes

- Care more about it being clean rather than certified
- But certification program is very important to commercial nurseries because they need those to be able to export

National Clean Plant Network (NCPN)

Grapes

- 2/3 of their production is grape-centered
- Wine industry in CA, they provide most of the clean plant material for the US
- Grapevine Virus Disease – what is the economic cost?
    - Done a poor job documenting the economic worth of their clean plant material work
    - Value of certification program in the North Coast of CA grape production is $60M a year
- Model and Reality of Grape Importation
    - Import dormant wood from Europe
    - Do disease testing
    - Disease therapy and re-testing
    - Provision foundation vines
    - Professional identification
    - Registered foundation vines
    - Go to nurseries and growers
    - *In theory can go for 2 years, in reality, goes to 9*
- Which testing methods?
    - ELISA
    - PCR
    - Woody field indexing
    - Herbaceous host indexing
    - NGS
    - Two are good – do we have to do five?
- Center directors need help to make this process efficient
- How much data is enough? How many publications? Which expert groups?
- State-by-State regulatory process is extremely complex. How about Federal Permitting as the easy piece of the pie?
- New Russell Ranch Foundation Vineyard, est. 2010, Davis CA
    - Microshoot tip tissue culture therapy
    - Negative test results – long list of pathogens – index, herbaceous, ELISA and PCR tests
    - *Agrobacterium vitis* testing at Cornell
- Why a panic about Red Blotch Virus?
    - Found red blotches on their grapevines that looked like leafroll at first
    - Zinfandel with Red Blotch – wine maker super stars, private consultants, commercial laboratories, the media, and a nightmare for growers and production nurseries

    - o Growers didn't want to pick up their vines in cold room storage until they had been tested for Red Blotch and freed of them
- Red Blotch Virus and Australia
    - o Australia tried to block grapevine imports from CA – regulators in Australia kept it open
    - o From experience, looks like regulators find every pest/disease they can and put it on the permits
- Germplasm and taxonomic collections – the key to microbiology

Is there a way to develop a sensible balance between scientific discovery, regulatory safeguard, and the need of commercial trade?

9:20

Dr. Ken Eastwell, Professor, Plant Pathologist, Washington State University

"NGS and Diagnostics in Current Practice (State of the Science)"

Grapes, hops, and fruit trees, the latter being the main part of his work.

1. What's the best available technology?
    a. Fastest, cheapest, or most reliable?
    b. Neither fast or cheap, very time-consuming program to test
    c. "Speed of Business"
        i. Time consuming program
        ii. Long timeline puts his business at a disadvantage
    d. Reliability
        i. Don't want to allow viruses in the country by sacrificing the long timeline
    e. Cost Effectiveness
        i. Some relatively modest cost savings available
        ii. If adopt NGS, save 50-100K a year
        iii. How much are we saving the industry though?
2. What are the limitations of NGS?
    a. Does NGS solve all of these problems? – No. But best technology we have available right now
    b. Is it truly a virus?
        i. Have to demonstrate graft transmissibility to confirm it's really a virus
    c. Sampling versus sequencing
        i. High throughput sequencing (NGS)
        ii. Propagate and grow in a plant to observe virus as well
        iii. Maybe not a sensitivity issue, but a sampling issue
        iv. Test budwood and plant material
    d. Data analysis
        i. Low titer virus – one size does not fit all
        ii. Need facility to do the backup work to verify or refute the NGS data
        iii. Be able to identify the results
3. What are the consequences of NGS?
    a. Outcomes of using NGS

i. Faster
ii. Reliable
iii. Does not rely on prior knowledge – huge advantage
iv. “conventional assay” development
v. New viruses – but are they new diseases?
    1. NAPPO list of 46 “pathogens” includes 27 uncharacterized “agents”
vi. Economic impact – direct and indirect
vii. Clean Plant Center vs. certification – how do we move forward?
    1. **Cautioned not to report some viruses because it could cause trade issues**
    2. **How do we report issues and deal with APHIS without it becoming a trade nightmare?**
    3. **We need a rapid and consistent response**
    4. **Need to be proactive to get their material tested with NGS before it has to get sent out**

9:43

Betsy Randall-Schadel, Plant Pathologist/Risk Analysis-USDA-PPQ-Science and Technology
“New Pest Advisory Group (NPAG)”

Mission: assess exotic plant pests that are new or not yet present in the US, but may pose a risk to US agriculture or the environment and recommends appropriate actions to the PPQ DA
Purpose:

1. An analytical process designed to provide pest information and recommend regulatory actions
2. Assesses biology, predicts potential destruction and spread
3. Inform federal decision makers

Scope: new and exotic pests

- Exotic – not yet present in US but a new pathway for introduction is identified

Functions

1. Evaluate significance of new pests
2. Coordinate information sharing and solicitation of expertise
3. Recommend options and actions on how to proceed
4. DOES NOT MAKE POLICY

Process

- Pest notification
- NPAG Core team
- NPAG report
- Recommendations
- PPQ Policy Makers

What qualifies as an NPAG pest?

- Is it a plant pest?
- Is it new to the US?

New Organism Process
- Identified through new tech
- Often no biological information for symptoms associated with the new organism
- Organisms may be potential pests, but clear evidence is often lacking to meet threshold for action
- Some organisms may be significant
- Critical to remain engaged with researchers
- Gather/track new information on organisms so NPAG can reassess significance if necessary

NPAG Report
- Brief direct report, use a template

Recommendations
- Add/remove pest from reportable/actionable list
- Go to different parts of PPQ

9:52

Heather Coady, Regulatory Policy Specialist
"Plants for Planting Regulation"

Current Regulation Categories
1. Prohibited
2. Not Authorized Pending Pest Risk Analysis
    a. Quarantine pest plants – list of species
        i. No import from all countries
    b. Hosts of quarantine pests
        i. Lists of genus/origin/plant part combinations
        ii. Import only from countries with significant trade history
    c. Allows to take prompter action
3. Restricted articles

Q56 (Fruits and Vegetables)

General Restrictions
- Import permit
- Port of Entry Inspection
- Phytosanitary certificate

Federal Orders for countries with significant trade history
- Emergency action to prohibit or restrict the importation of a plant
- Allows for risk mitigation measures

Controlled Import Permits (CIP)
- Created consistent set of conditions for the previous departmental permits
- Limited to restricted pant material for experimental, therapeutic, or developmental purposes
- Requirements for growing and release
    - Containment
    - Inspections

  - Small import quantities
  - Each new permit limited to 1 year, 1 shipment, and 1 country

Fruit and nut plant regulations, NAPPRA, and CIP
- Not all fruit and nut plants are prohibited
- All fruit and nut plants require post-entry quarantine

Basics of Rulemaking
- To tell the public what we're doing
- Give people a chance to respond – respond to every comment/question

Q37 Restructure
- Add requirements for IPRMM

10:10

Shailaja Rabindran, Assistant Director & Plants for Planting Team
"NGS Procedures, Practices, and Policies"

Current requirements of importation of prohibited propagative material for research, therapeutic, or development purposes
- Material can be received through PGQP Bldg 580

Limitations of NGS
1. Dependent on pathogen sequences in databases
2. Provide bioinformatics information__

Policy issues
1. What is the scope of plant pathogens and plants covered by NGS?
2. Role of NGS in
   a. Certification and quarantine programs
   b. Diagnostics
   c. Replacing existing technologies
3. Transitions
   a. Research, policies, procedures, facilities, and resources needed to transition to NGS?
4. NGS protocols/methods
   a. Standardized, validated and approved?
   b. Which labs will conduct these tests?
   c. What labs will confirm results?
   d. Supporting evidence for negative results?
5. Novel sequences
6. International acceptance and trade

Policies/procedures for the discovery of "novel" or unknown pathogens
APHIS response includes
- Policy management: imports, domestic programs, NIS PIM
- Science and tech
- Field Ops

10:20 – Break

10:50

Panel Discussion
Dr. Ken Eastwell
Dr. Joe Foster
Dr. Deborah Golino
John K. Greifer
Dr. Shailaja Rabindran
Dr. Nicole Russo
William Thomas
Betsy Randall-Schadel

For Ken Eastwell: presence of a lot of historic pathogens in quarantine programs. Certain pathogens are an expression of multiple unknown pathogens. Is there an expedited way to drop these historic regulations?

- List of material available of + controls for NGS. Strapped for resources.
- Regulatory perspective: hasn't progressed that far, still looking from practical perspective
- Consider writing a chapter for a book on phantom viruses – documentation there
- Not on quarantine list – won't do anything about
- Might be finding pathogens that should be on the list though
- Program to look to de-regulate pests
- Virus collections disappearing making their job harder
- Sorting different strains that cause the particular disease is very time consuming and expensive
- Multiple viruses that cause diseases – multiple disease names for the same pathogen

IPPC – some countries already using technology for other purpose: which countries are using it, what are they using it for?

- We have exported to New Zealand – approved our protocols for export to NZ. Not part of their official quarantine program, but researchers use it for material still in quarantine
- Not as an official regulatory tool, but in use

Export requirements oftentimes ambiguous – photos are the most extreme cases, what about looking for single isolates?

- Another country is mandating, we can work to communicate with that country's QP if we can prove those pathogens don't exist in the US

For John: Are there NGS related standards being developed at NAPPO levels? Proper/appropriate use for certification?

- Short answer's no. Too cutting edge, hasn't come to the regional plant protection convention
- Technical panel (diagnostic) to look at tools for irradiation
- Can see it down the road – does become a regulatory tool to be discussed

- Have to be transparent about how and what we do that will inevitably have countries work together to not disrupt trade

Can we say "we can't test for X pathogen" if we don't have it?

- Countries can negotiate with us for us to convince them X pathogen doesn't exist in the US
- New commodities

NGS: is the only place that clean plant center protocols are approved on a controlled import permit?

- Eastwell: Our CIP covers foreign and domestic protocols.
- Foster: for centers, they get a permit with a list of what tests they want to do on these plants; once they get this permit can get their plants from anywhere and carry out their tests. States are free to set their own incoming standards

If in fact the control import permit is really designed for international movement, do we have an opportunity to specify protocols that we approve that you want domestically, that includes NGS rather quickly.

- Golino: opportunity to test the waters with it.
- Eastwell: I mentioned yesterday we had a situation with grapevines where we don't import material. All our material is domestic. Therefore, I'm not required to use NGS for importation, but the state certification programs beat me up for that proposal because they want to export to Canada and they don't use NGS

Is there talk about opening up labs for public controls?

- No discussion right now, but addressing issues with seed commodity. Currently getting ready to start a private program to accredit private lab testing
- Foster: Can the new person (private lab) who wants to do this duplicate what others are also doing?
- Send in proposals and protocols

Recent evidence that seed transmission did occur: those reports resulted in a lot more investigation, providing enough data for us to say "no this is not a pathway" and de-regulate it. It happened very quickly. Pulling stakeholders into the process makes things move very quickly. Accreditation and certification of programs that may be very helpful. Comments about true validation of the methods, making sure you take steps to ensure variations of the method are equivalent. Requires significant investment and resources. NCPN is talking about lab accreditation, this is no different, just maybe little complicated.

Most immediate needs were making sure some labs were up to snuff (autoclaves, re-sheathing greenhouses, staffing), and now the network has 26 programs (started with 4). Immediate needs are being met, now looking at signature plan, certification and quality, interstate movement of materials. Members of the clean plant network engaging in coordination.

Accreditation of private labs: somebody has to set a policy

- Control import are not for domestic material

- Movement of domestic material and certification: between the states there are so many requirements and that needs to become more consistent.
- Varies country by country who accepts what type of material

Export, domestic movement: National Certification Program is what we need

- We don’t have it.

Country that requires testing for a pathogen we don’t test for: we can send material to private companies and get the results, and our nursery inspector can sign the phytosanitary certificate

CFIA in Canada can accredit private labs to do testing with some programs. Protocol for it, labs have to have quality management systems in place and demonstrate they’re able to do this testing.

NGS: a wonderful tool that can solve a lot of problems with long-term indexing with woody plants. How do we handle the viruses that have existed for a long time that we’re just finding? How do we address them and make a path forward for each one of them? Industry perspective: can be understanding if progress is being made and decisions are made for a reason. But if nothing progresses because someone found a virus that no one’s heard of before there can be a lot of frustration. Want to address how regulatory people and scientists can form some kind of quick strike committee to address these things as they come up. Key people in APHIS and scientists, if we can just address them individually and develop a plan to move forward that would be very helpful.

- Problem is what do we do in the meantime? Has to be a way of quickly getting guidance on how these viruses are managed until we get all the biological information.

In fruit trees, therapy techniques can work well on certain generates, but not on others. Most apples can go through the program in one year. Not the cherries.

What we have established is not serious. We may have a few exceptions. On cleaning up, we need to have it as clean as we can. There are situations where we may find out it’s impossible to have it established clean. If something is moving out in a field, we have a large amount, if every rootstock is infected, it doesn’t matter that the scion was clean, what do we do?

2nd generation NGS: 1st generation is less material, but has the potential to be very simple, fast, and cheap.

- We’re just talking about different bench tops, 1st, 2nd, and 3rd generation NGS is really the same thing just different bench tops. Also user friendly. We need to consider you need to work with a good company with a good support.
- Putting on the regulator hat.

12:00 – Lunch

1:00 – Roundtable Discussions

Group A

Questions:

What are the next steps for the scientific community to take in order to move forward (top 3)?

Best practices, analyzing data

When something happens, we don't know what to do next? Need a roadmap for how somebody navigates the APHIS system

Goal: Move forward using NGS as an APHIS-approved diagnostic tool

1. Development of data to prove NGS works equally well or better than the approved and current method
2. Develop minimum guidelines (NPAG) for reporting a new virus-like pathogen
- When we use NGS for diagnostics and find something new, you should follow the guidelines
3. Develop a database of regulated pathogens per taxa
4. Scientists to submit the proposal for NGS to be sufficient to qualify items for provisional release
- Use the standardized format already in use in APHIS

Identify taxa to understand how much time should be allowed for each

Collaborate with trade partners and trade directors in APHIS

Develop a streamlined process to achieve main goal (scientific roadmap)

Market new technology to stakeholders

How do we take data for new pathogens? How do we communicate with APHIS?

- Anything involving novelty should be verified via graft transmissibility
- Press release about meeting and what is happening (when approved)
- How will we communicate with the public? Deliver the message?

Gain support from the trade group

NGS will show us there's a whole lot of microorganisms we didn't know were there previously.

Reaching out to other groups (food and animal, not just plants) because they might be farther along than we are and could be good resources.

What are the next steps for the regulatory community to take in order to move forward (top 3)?

Goal: To accept NGS after receiving sufficient information to move forward with approving NGS as a method with confidence

1. Include colleagues in industry and states in a continual conversation
- Commodity expert groups? Who makes up the continued conversation group?
- Federal, State, and Industry
2. APHIS needs to make a roadmap as well of the process
3. Evaluate proposals (Deb) submitted
   a. Prepare to receive proposals – work group, task force, whatever to expedite the process

4. Communication – what will it look like internally

Need to see all the data/validation process
- Mechanism for validating the data they're using

If you see the data how would you validate it? Compare from other processes
How can we use each other's data to validate and verify?
Different criteria for different taxa on a case-by-case basis
- Identify the taxa (pathogen/host) that will be NGS approved
- What needs to be communicated and to whom? (White paper)
- Internal dialogue/conversation and then reengage scientific community

Immediate next step: talk together and make sure they're on the same page
- Enlarge the conversation to re-engage the center directors/states/etc.

Group B

Questions:
What are the next steps for the scientific community to take in order to move forward (top 3)?

What are the next steps for the regulatory community to take in order to move forward (top 3)?

Goal: Engagement of scientists with regulators
Taskforce(s) that includes PPQ, loosely WERA-20 (research, industry, state-level regulators) to talk about some different things
Tasks:
Form "taskforce(s)" – PPQ, NPB, Univeristy, WERA-20, Industry
1. Lay out process to set validation requirements to incorporate NGS into existing programs
    a. Gather data in one spot
    b. Weigh data to have evidence to know what's real and what's not
    c. Update many specialty crops – lists of viruses in a particular host to include NGS in the matrices and make them available for anyone
2. Develop the path to creating standards for NGS-type testing by crop, template, virus, what kind of internal controls do you need, etc.
    a. By crop/template/virus/spiked controls, etc.
3. Develop policy guidelines for initial strike team for novel find review
    a. Building nucleic acid libraries from around the country (find a novel sequence you can do a snapshot to see if it's widespread or not)
    b. NPAG Form, moved beyond NPAG
    c. Transmissibility is key

Develop standards for NGS testing
- By crop, by template, by pest?

Validation and side-by-side comparisons → ask CPHST for collaborative effort

Taskforce → Identify available data

- Get it to policy people
- Guidelines for initial review/recommendations for novel finds?
- NPAG form with info on where problem movies if NPAG is not able to act yet

Build N.A. 'libraries' to quickly test when a new virus is found

Matrices for crop/virus/detection techniques to include NGS

- Categorize "agents" known-unknowns-quarantine
- Make readily available

DepAdmin, WERA-20, NPB → Form/request joint taskforce to determine validation requirements for NGS

- Research, government, industry

2:45 – Break

3:15 – Report Out

3:30 – Closing Remarks

WERA-20 Meeting
July 8, 2015
Day 3

Research Updates: Clean Plant Center Northwest Updates
Ken Eastwell

There has been a severe drought in Washington State, not as bad as California (4-year drought), this is the first year of drought they've had. Increased temperatures have caused viral activity to spike. One of their biggest issues is Little Cherry Disease. They've had to remove 450 acres last year because of this disease. RPA technology is promising for virus detection. They ran into some problems with false negatives, though, so they had to do some optimization. Timing of the exam became key: they found dormant budwoods were okay, then petioles after the leaves turned straw colored were good. One of the major pathogens they have had since 2000 is Western X. Found WX is transmitted through leafhoppers (mountain, sharp-nosed, and cherry leafhoppers). Don't know the economic risk of WX yet. As of the end of January, the Clean Plant Center has been made official.

Questions:
Clean Plant Programs: where do the new selections come from?

- Varies with each specialty group
- Proprietary -88-90%
- Hops: NCPN – ask for nominations for industry and we select which ones will go through the program. Have to justify what the economic impact of the hops is.
- Grape: separate advisory group – ask for nomination from pacific NW, group meets and identifies selections to bring into the group. From industry
- Fruit: we make some decisions based on public varieties we need to replace

Is there cross-protection between LChV1 and LChV2?

- No, symptoms tend to be much more severe with LChV2 when it's alone. LChV1 has mild symptoms when alone
- Only see LChV1 when in combination with other pathogens,

Do we have Peach Leafroll in WA?

- No

Do we have anyone working on the alternate host for WX program?

- Don't have a lot in the state in dandelions
- Have entomologist on staff working with Dan on sweeps in cherry orchards every 2 weeks. Have sticky traps looking for leafhoppers. With those we can see if they're carrying them

Can NGS detect phytoplasma?

- Yes

RPA used in diagnostic labs, or can be used by field men?

- Intent is to be used by field men.
- When some field men use it, results couldn't be duplicated from the research lab. With some experience we could get there, that would be ideal to have field men use it.

Material arriving at Prosser is generally cleaner than when you started doing this years ago?

- 40-50% of trees coming into our program are infected

Washington State Department of Agriculture
Lauri Guerra

Systemic problem has arisen in rootstocks that goes beyond testing. In the beginning we found cherry orchards had a high number of infected trees. Commercial rootstock had been tested for ilar, less than 5% appeared. Peach trees are struggling. Pome trees: start clean, stay clean because there's no movement of the virus where we are, but not the case in reality. 2001-2002, found CLSV, and found the problem was in the rootstocks. We found four blocks at a nursery which had a problem, and one whole row was infected and shouldn't have been there in the first place. We restricted our nurseries, designated rows to investigate more often, but even that was challenged. After testing for three years, they had one row and wanted five to test; they planted a whole new block. It appears the CLSV is moving. Started finding _CLSV_ on both mother plants and rootstocks, but on Russian, saw symptoms of CLSV on uninnoculated plants. Checked in the greenhouse, got mother plants in tissue culture. Went through his list of nurseries and saw a lot of trees were infected. It's a big problem, have to approach the entire thing differently, and not with more testing. The system we have been using is a failed system. Now know we have to start with TC at G1 then move up to G2, G3, and G4, and if it fails in G2 it has to go back to G1. Can't keep repeating the same mistake. No source of G1, G2 rootstock available.

Questions:
Has it been discussed as an action item in WA?

- This is the first time talking about it openly in a big group, and definitely something we need to resolve together.

University of Hawaii
John Hu

Hawaii is paradise for people as well as viruses and pathogens. Banana bunchy top virus (BBTV) has been found in a lot of Asian/Pacific countries and now African countries. Transmitted by banana aphid and is quite an old disease. It has at least six DNA components. Now we know the different functions of each component. We have been trying to produce transgenic resistance of BBTV. On Component 1: transformation of embryo genic cell suspension. Screening for BBTV resistance by collecting aphids from diseased plants and putting them on young banana plants (~1 foot long), and found 65% transmission. Put at least 10+ on each baby banana plant to get 100% transmission. In molecular screening, they can run a gel and see plants with the BBTV and run them through TC, then test with PCR to see they are still transgenic and have the target gene. 17 lines have been tested; 3 lines are in the field now. 6-8 plants were in each line. Grad student ran BSV (Banana Streak Virus) testing with RT-PCR and RT-LAMP systems to detect presence. It is climate sensitive. Combined tests to find any other

presences of virus. Transgenic papaya in Hawaii – were able to send seeds to China to conduct field tests. China wants 1,000kg of nontransgenic papaya fruit.

Questions:
Were you looking for the expression of transgenic in the RNA?
- We didn't expect to have any protein produced
- We did not do laboratory expressions of RNA

* What was the sample size you started with?
- We obtained 200 lines, only 20 went into the greenhouse, put in TC, propagated, and then put into the field
- In greenhouse, those 20 trees didn't show any symptoms, but in the field, some of them showed symptoms
- Based on molecular biology, we only have 2 strains: Pacific and Asian strains

Reason for failure possibly gene silencing?
- For other single-strand DNA viruses, people have different results for gene silencing
- Haven't found anything reliably resistant to BBTV

Grapevine viruses in Idaho
Alex V. Karasev,
University of Idaho

2K acres of grapevine. Visual cues of virus presence. Got 434 leaf samples from four counties, and did over 6900 RT-PCR tests, finding 16 viruses and viroids. Most frequent positives were GLRaV-3, which is transmitted by mealybugs. Our recommendation to them was to control the mealybugs to control most of their other problems. Part of developing ELISA-based diagnostic assays most convenient methodology for large-scale tests for grapevine viruses. Produced leafroll1-specific antibodies against recombinant CP. This TAS-ELISA test is sensitive and specific. Working on red blotch in Idaho. Effect of red blotch on grape quality – comparing data to see if there's a significant change in quality because of the virus.

Questions
How do you do quality checks and how do you control variation of berry quality? How do you sample?
- Colleague is a food scientist: looks at sugar quantity, etc. for berry quality
- Collect multiple clusters of grape, look at yield, and analyze the composition of berries. We do it for more than 1 season.

Can you define healthy plants? How many viruses have you tested for those healthy plants?
- Tested for 17 different viruses/viroids
- Healthy in the sense it is red blotch negative
- Healthy based in appearance in one particular case.

What do you recommend to growers if they have bad leafroll?
- Biggest problem in Idaho is Leafroll-3 and it's moving relatively fast
- I recommend replacing it

- Too early to make recommendations on red blotch – there's no consensus in the community about it yet

Transmission happening and going into younger vineyards, but it's all Leafroll-3, we do have some areas for red blotch where it is moving and others where it's not.

By consistently providing clean material, you eliminate non-transmissible viruses and put more pressure on transmissible viruses to expand.

10:15 – Break

10:45

Characterization, detection, and *in vitro* elimination of viruses infecting fruit trees
Dr. Ruhui Li
USDA-ARS

Apple green crinkle disease (AGCD) limited to apples. Infected plants never produce any fruit, no fruit symptoms. Literature suggests some strains of ASPV causes disease, so they compared two groups and haven't found it, therefore cannot make a solid conclusion. No new viruses were identified from both trees, but multiple variants of ASPV were found. Detecting APLPV in *Prunus* quarantine in Bldg. 580. Multiplex TaqMan RT-PCR developed for detection of 3 viruses; very specific, sensitive, reliable, and rapid. Want to reduce the time of *in vitro* culture. Efficiency: by using heat treatment, especially in higher temperatures, 100% inactive plantlets, but only after one run through the PCR. The virus was suppressed, not eliminated. Shoot tips from axillary buds were better starting material than axillary buds. Two runs of heat treatment are more likely to be more effective than one.

Questions:
Plants negative on first test: go over into dormancy and then treat them?
- No, they were just discarded, but the bulk of the plant were kept in the greenhouse

Effect on plant growth from the chemical therapy?
- A lot of effect on them: some were effective for virus elimination
- Size of explant determined level of toxicity

Blueberry Fruit Drop Disease
Oregon State University
Bob Martin

Blueberry Fruit Drop spreads slowly and is now in adjacent fields, but is symptomless except in cultivar 'Bluecrop.' Occurs near Olympic National Park. 'Bluecrop' the berries fall prior to harvest. Does not cause any disease or cause a synergistic reaction; right now it's a

nonissue. Fast forward 8 years: found very good correlation with fruit drop symptoms and positively tested plants for BFDaV. Pretty sure we have a causal agent. Trying to find a host for the virus. Will retest the field spread annually. Duke is susceptible to virus via grafting. *Rubus yellow net virus*: not graft transmissible. Found a graft-transmissible isolate. Raspberry Leaf Curl Virus transmission by *Aphis rubicola* but not mechanically transmissible. Testing is required for plant export established in 2012.Looking for RLCV. Found two carlaviruses in Wisconsin. No type isolate for RLCV. Blueberry Shock Virus in cranberry detected in different states in 2012-'13. Pollinated them with blueberry shock-infected pollen from blueberry plants, took them much longer to flower than the others. Blueberry Latent Virus no longer on the quarantine list. GRBaV moving very slowly in one vineyard in Oregon, but almost no Leafroll-3 in the vineyard. Using pheromone traps: might only get one mealybug all summer.

Questions:
Blueberry Latent Virus lacks
- Seed transmitted

What does it mean if you pollinate cranberries with blueberries?
- Pollen will go into the stigma and will transfer into the maternal tissue and transmit the virus that way

Aphid transmission of RLC: persistent, picked up in short feeding periods, how short?
- An hour
- Raspberry latent replicates in the aphid and is transmitted persistently.

New Hosts for Hibiscus Green Spot Virus 2

University of Hawaii
Mike Melzer

Hawaiian leprosis – orange ringspots on stems, seeing symptoms on leaves as well. Infects *Hibiscus arnottianus* too. Tri-partite genome: triple gene blot, maybe that's why we see the lesions expand on the leaves. Non systemic, just localized lesions. Does it naturally infect economically important citrus? Leads to early defoliation. Farm in Maui: No lesions on the stems though just leaves; it affects nearly 100% of the leaves in citrus trees. Don't see necrosis on Hawaiian leprosis, just chlorotic. Symptoms associated with HGSV-2 infection. Some argue it's a mite virus. HGSV-2 spread from original volklemon tree in Waimanalo to grapefruit, navel orange, and mandarin.

Questions:
How close is it to green spot?
- We don't know. Called it Hibiscus green spot first because it produces an ornamental hibiscus similar to that in Brazil, but found it's a different virus altogether. No sequence information. Is a virus, but no genetic information
- HGSV-2 is tripartite
- Probably not spread by budwood – slow spread

Affect fruit quality?
- No not really, not as much as the Brazilian green spot virus

11:50 – Lunch

1:00

National Clean Plant Center for Berry Crops at NC State University
Zvezdana Pesic-VanEsbroeck

Have been doing sweet potatoes since 1988 but are now entering the Clean Plant Network with them. Currently working with sweet potato, strawberry, blackberry, raspberry, blueberry, and muscadine grapes. Want to join the NCPN for grapes next year. Want to produce, maintain, and distribute pathogen tested G1 plants. Funding comes from private sources and some from the university. About a $500K budget, smaller than most other places. Large blueberry program at NCSU. Production of G1 Foundation Plants protocols include testing material for viruses by PCR, ELISA, dsRNA and biological indexing. Very careful when people come to receive plants – they need to have all the correct paperwork. Virus testing for Blueberry Latent Virus: 50% of incoming material has it. Plant material distributed to nurseries includes *in vitro* and potted plants. Outreach includes open houses, poster presentations, tailgates (non alcoholic). Impact of MPRU Strawberry Program: tens of millions produced each year in NC. G1 plants are free of economically important diseases.

Virus Survey in USDA Berry and Hazelnut Germplasm
Corvallis, Oregon
Joseph Postman

Pyrus over 2K accessions coming into National Clonal Germplasm Repository in Corvallis. *Rubus* taxa over 2K accessions, 1400 seedlots 900clones. A lot of interest in heirloom varieties. Interest among plant taxonomy for clonal identification or taxonomic identification. Testing by ELISA for ApMV, RBDV, TobRSV, TomRSV. 47 out of 907 accessions were positive. Bob Martin is in the process of examining 92 accessions for new and emerging viruses. 1700 *Vaccinium* accessions in 952 seedlots, 310 blueberry clones in the field, and 765 in screen/greenhouse. Pollen borne viruses are moving rapidly through the area. Blueberry shock virus as well. 300 clones in field collection being tested for BlScV and BlShV: Blueberry Scorch not detected so far. It’s an aphid-borne virus. 850 clonal hazelnut accessions representing 20 taxa from 38 countries (no seed accessions). Only ran ELISA for ApMV. Successfully eliminated it from 25 variations. Haven’t seen much spread in berry collection for those that tested negative in the past. Low level of viruses in new introductions.

Virus Survey in Pome, Stone, and Small Fruit Nurseries in Oregon, 2014
Oregon State Department of Agriculture

Dipak Sharma Poudyal

Major producer of fruit tree planting materials: official testing program for blueberry, and certification program for pome, and stone fruits as well as grapes. Tomato Ringspot Virus in Malus and Pyrus: 23 nurseries in 2014, sampled 7.5K with ELISA and got all negatives. Also looked for latent viruses in that sample set for ACLSV, ASGV, ASPV, all negatives as well. Tested stone fruits for Cherry Virus A – 14 nurseries, 312 samples, with RT-PCR and found 18% of samples were positive. Blueberry: tested 10,325 samples for BlShV and BlScV. All negative for BlScV, but 6% positive for BlShV. TbRsV/ToRsV transmitted via nematode. Took soil samples from 15 nurseries and found no parasitic nematodes

Questions:
What do you mean by samples?
- Collect one stick and one leaf from 4 different trees in the plot and make them into a composite one sample.

Do you do any PPV testing?
- Yes, but not counted as part of our certification program.

Nursery material?
- From G2 and G3, whatever they produce is what we use for testing.

How do you trace back?
- We have a system: we are able to know exactly where that sample comes from.

Virus diseases in Washington State Vineyards
Naidu A. Rayapati
Washington State University

2014: ~50K acres of vineyards and ~850 wineries. Riesling, Chardonnay, Cabernet Sauvignon and Merlot major grapes. Almost $8B economic impact statewide and almost $145B national economic impact. Looking at host-virus interactions, education and outreach, impact of viruses on fruit yield and quality. Leafroll disease is the most severe problem in his grapevine production. Fanleaf degeneration/decline causing smaller fruit clusters. Sent samples to Agdia, results showed they only tested positive for TRSV. Collected berries from symptomatic and nonsymptomatic trees and examined quality. TRSV spread by nematodes? *Xiphinema americanum* found in vineyards. GLFaV-3 predominant and widely distributed. Red blotch less predominant compared to GLFaV-3. Hard to differentiate between what is leafroll and what is red blotch due to lack of symptoms. Diagnostic assays should be used.

Grapevine and Tree Fruit Update
Canadian Food Inspection Agency
Anna-Mary Schmidt

Grapevine virus survey in British Columbia. Samples come from CIFA-certified foreign services, as well as a breeding programs and nurseries within the country. PPV surveys to establish presence of particular viruses in Canada. Some plants came through border inspections and nurseries. Heat therapy and TC for virus elimination. Still in possession of repository, will become increasingly important. Material is required for certification program but also available for growers. Trend over the last 5-6 years is fewer detections of leafroll. Canada doesn't have a small fruit testing program, but they do a fair bit of rubus testing for the US. Two detections with red blotch in BC. Want to integrate NGS as a routine diagnostic tool. Want to expand commodity range to develop a national certification program for berries and increase virus elimination capacity.

Questions:
Source of non-certified material?
- Non-certified is from anywhere, has to come directly to our postentry facility though.
- Non-certified means CFIA hasn't certified, but Canada might not have

Red blotch is in BC?
- We don't have all the data for that yet, but Mike is working on it

Michigan Small Fruit Virus Update
Michigan State University
Annemiek Schilder

Over 20K acres of blueberries and grapes each. Nepo-viruses, sandy soils = a lot of nematodes. Found 42% of samples were positive for up to 4 or 5 viruses. Leafroll-3 most common. Chardonnay vineyard – found a lot of Leafroll-3 and TRSV, some in combination. Severe winter: low yield. 85% of vines had Leafroll-3 symptoms. Samples show the highest identity to a TRSV isolate from a soybean in South Korea. Had a lot of grapevine mealybugs. Have to determine how widespread the mealybugs are. Peach rosette mosaic virus sporadic in old Concord vineyards. Niagara grapes: shiny leaf disorder – unknown cause. Makes clusters really small. Varieties are non-symptomatic, makes it hard to test. Bronze leaf curl – seen in many older fields in a lot of varieties. Kills bushes. Some spotting on blueberry bushes, on the top and bottom of leaves as well as on the stem. Not spray injury. Wavy line ringspot – fairly new. TRSV in raspberries.

Questions:
See on the weeds symptoms for tobacco ringspot?
- Some symptoms on the weeds

Are you able to get a clue ahead as an indicator or only see things once they've happened?
- We've thought about it
- Testing dandelions. 50% come out positive.

What action will you take in the weeds?
- We recommend 2 years of weed/grass crop with strict broadleaf control.
- We've had some blueberry growers who wanted to fumigate but it was too expensive and remote.

3:15 – Break

3:30

University of Arkansas
Ioannis Tzanetakis

A new badnavirus infects blackberry and is present in all states. Not an accidental infection. Fairly easy to move mechanically. A lot of infected plants that give no symptoms. Genome organization is pretty similar to other badnaviruses. Integration: more than one genome present. Elderberry carlaviruses A-E: new discovery. In 5 plants, there were discovered 5 viruses and a sixth new one. Viruses A, B, and D go with ApMV, and viruses C and E go with another replicase. NGS mapping and PCR could not identify isolates EVA and EVB in Maxima.

Questions
Badnavirus: is the isolate integrated?
- Having the extra bands would show that it's integrated

Would it be possible to not even digest it? Sometimes you get a band up top and then a badnavirus on 7KB
- This is digest control
- There are two negative controls, but when you keep it and digest it, the two bands up top show up

You have your herbaceous uncut?
- Yes
- We digest before we do the RT-PCR

PDA Fruit Tree Virus (mostly) Programs
Pennsylvania Department of Agriculture
Ruth Welliver

Same mission as USDA-PPQ at state level. PA Fruit Tree Improvement Program. We audit a system put in place to keep the clean plant from Prosser clean until they get to an orchard setting. Keep up communication with the growers. Test for PPV, PDV, ToRSV, PNRSV and nematode vector *Xiphinema* sp. 2014 Orchard Exotic Disease Survey including PPV testing. 2014 Exotic Disease of Orchards, including exotic phytoplasmas and exotic *Monilinia* spp.. Found some pear decline, but two apple trees demonstrated signs of a new disease they named Apple X-Disease. 2014-15 CVA Survey participant: sent samples to Prosser to support their survey. 2014 Apple Orchard Decline Project: winter injury? Residual herbicides? A lot of apple orchards roughly 6 years old with apple trees declining that's killing the orchards to 50% dead. Tested for ToRSV, ASPV and ASGV. Every site is a little different, no consistent finds across sites. Spotted lantern fly: new moth discovery posing a large problem.

Questions:
Apple X-Disease: seen this in subsequent years?

- Saw first in 2013, then 2 trees in 2014, asking the people to remove the tree in 2015

Development of Novel Isothermal Amplify RP for Rapid Detection of Plant Pathogens
Agdia, Inc.
Shulu Zhang

AmplifyRP: targets a template with two primers, an internal probe and a recombinase; amplifies the target with a polymerase at a constant temperature rapidly. Two formats: Acceler8 and XRT. Acceler8 uses an endonuclease to cleave at the THF residue to produce a dually labeled DNA amplicons. Qualitative assay, contamination risk is kept to a minimum. Two methods: combo AmplifyRP and Multiplex AmplifyRP. The first one uses real-time and endpoint product assays using a single common reaction, while the second one has two sets of primers and probes. Applications can be used real-time and endpoint assays can be qualitative and quantitative; can be performed in the field or lab, and can be used in testing multiple samples quickly and simply.

Grapevine Red Blotch
USDA-ARSUC Davis, CA
Mysore Sudarshana

Started 2008 – Leafroll-3 and red blotch have the same type of symptoms – make it hard to differentiate. Drought has exacerbated the red blotch. Red blotch virus found in 2011, announced in 2012. The red blotch can sometimes show symptoms and sometimes not. Have to really test it. Can tell a difference in the wine if the grapes had red blotch or not just by the smell; they look the same though. Red blotch wine sells for $10,800 revenue/acre vs. 90K revenue/acre for healthy grape wine. Incidence of red blotch as high as 100% in blocks.

WERA-2- Meeting
July 9, 2015
Day 4

8:00 – Tour of Inspection Station at Building 580

9:15 – Tour of Containment Unit at Building 580

9:45 – Tour of CPHST at Building 580

10:15 – Break

10:30 – Welcome and tour of PGQP at Building 580

12:00 – Lunch

1:00 – Housekeeping announcements

1:10

Business Meeting – Hanu Pappu

Where do we go from here?
One proposal for hosting the WERA-20 next year: Deborah Golino at UC Davis

- Opened a second facility at FPS with a big conference room
- Best time of year for symptoms is the last week in September.
- 8-acre virus collection, singly and multiply-infected
- 3rd week is the latest federal employees could get leave (and money) to go. Second and third week would be better
- Deborah will talk to Dr. Moyer and see if it'll work with his schedule.
    - Wants to give people a hard 20 minutes for their presentations, 15 for speech, 5 for questions
    - Please register early even if you can't pay early
- Graduate students permitted
- Would have facilities not accessible by walking – would need cars/shuttles
- Two days on campus, second morning/afternoon for local fieldtrips

Other new people coming into the field – start including a wider group

- Intentional and strategically reach out to other colleagues

No one objects to having their PowerPoint/reports shared through private email to people from the meeting

- Objections to having it on WERA site

1:30

Special Topics
Germplasm-Born Pathogens

Joseph Postman

1983 Ad Hoc Committee Recommendations
- A lot of the same language that have been used this week
- Materials introduced from outside US conform to APHIS quarantine regulations
- Germplasm repositories implement explicit therapy measures, in cases of known viral contamination
- Wherever possible tested, disease and pest-free materials should be preferentially selected sources for plant introduction

Plant breeders a part of the problem of virus spread
Present situation at fruit ad berry genebanks
- Genebanks for crops with very high-risk pathogens have thorough testing and therapy programs (i.e. citrus)

High requests for these crops that should be kept in genebanks:
- 'Olympia' blueberries of no really great importance
- Cider apple 'Ashmead's Kernel' used for apple cider and hard cider almost all have apple mosaic virus
- 'Marshall' strawberry – not very durable, but have sentimental value and are still requested

What should USDA genebanks look like in 30 years? How do we plan for the next 30-40 years?
Who is going to maintain virus collections?
Sequencing will provide simultaneous pathogen detection and identify verification
- Maybe having a reservoir of heirloom varieties with their viruses isn't that bad a thing

Questions/Comments
NCPN money needed to keep cleaning up material
Visited Institute of Fruit Research in Japan: original green core and Fuji apple trees that are still maintained. Have an orchard to maintain all the apple trees.

Is there a clear separation of things you're saving for virus isolates or for cleanup?
- Some are isolates, separate collection
- 9K and greater is virus isolates. Anything 1+ is Germplasm

Preserving some specimens of Joseph's for freeze-drying

Consider a G2 block for NCPN?
- Ken Eastwell: We've always done that in the past. Don't want to discard trees

580K plants in the National Plant Germplasm System: if we were to start saying we were going to use NGS on these collectionswould appeal to curators a lot more if it also helps them rationalize collections, eg. identify duplicates.What are we going to do if, for example, we find out 75% of our collections are infected with one or more pathogens?
- As of right now we don't know if they're all pathogen infected

- Groups of people using sequencing rather than smaller random repeats for getting fingerprint clones.

Knowing the origin of a pathogen is really important

Europe has a complete quarantine on imported blackberries because of Xylella

Teaching Virology
Annamieke Shilder

Students don't learn virology – just plant pathology
What is the future of teaching virology? Is anyone teaching an online virology course? What about virology lab component?

How can we make virology a wider-taught class?
Guest speakers?

Connect with Hanu Pappu for recorded lectures as a resource for virology courses

USDA should team up with University of Maryland to teach virology
- Students come each year to work with APHIS

Sending students away to be exchange students to take virology courses?

Reluctance to hiring more teaching faculty

University of Hawaii – opening up a new set of classes students have to take. A master's program without the master's thesis
University of Washington same thing: 40 weeks long. All students expected to take it. No labs, just classrooms.

2:40

Expectations of where we will be one year from now

Expect to accomplish?
- Response: Can't do anything until we hear from the regulators.
- : It is up to the scientists to make the proposals to incorporate NGS and then defend using science to convince the regulators that changes need to happen as a consequence of the new information in existence. Then we can expect policy
- If we put in an application for controlled import permits (CIP), they'll look at it and it'll challenge them to develop a policy
- We shouldn't wait until we know everything we think we need to know: we should do it just like what we do with a grant proposal – put it in and see if you can get it.
- If you want anything done, scientist must continue to be in contact with the policy group of people we met in the first two days and keep the channels of communication open.

Request form policy a roadmap, provide the science to policy so that they can do the necessary changes to the policy. Seek the support of the industry you work with. This will make the petition stronger and the process has a better opportunity to take off.

How can we move material across lines better?

- First you have to send them a note to inactivate the virus from the regulated quarantine list
- Keep asking if you haven't heard from them
- Try to get NGS to be a routine tool used by APHIS – if you can do that a lot of other places will follow suit.
- Shouldn't have to get back to regulators every 6 months with follow up questions
- Push shouldn't come from the scientists (they're providing information) – should come from the importers

Quad: NZ, Australia, Canada, US – need annually to discuss issues of common regulatory concerns.

3:00 – Closing Remarks, End of WERA-20 Meeting