siRNA sequencing and assembly: reconstructing viral genomes from defense molecules

Jan Kreuze
Next generation sequencing for viral diagnostics

- Next-generation sequencing technology is promising universal sequence based diagnostics
  - Total RNA (for everything, but lots of sequence ‘wasted’)
  - dsRNA (not all viruses very well)
  - Virus purification
Next generation sequencing for viral diagnostics

- Next-generation sequencing technology is promising universal sequence based diagnostics
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  - dsRNA (not all viruses very well)
  - Virus purification
- Small RNA sequencing and assembly: sequencing the anti-viral defense molecules
Anti-viral RNA silencing in eukaryotic organisms

- **Nucleus**
  - DNA-virus (e.g. geminivirus)

- **Cytoplasm**
  - SiRNA
  - Maintenance, amplification
  - "Degradative PCR"
  - RdRp
  - Rnase
    - Initiation
  - Targeted RNA degradation
  - Overlapping ssRNAs
  - dsRNA (RNA virus)
  - Replicating RNA virus
  - Complementary ssRNA or dsRNA
  - Systemic signal
  - RISC nuclease complex
  - "Degradative PCR"
  - Maintenance, amplification
  - RdRp
Anti-viral RNA silencing in eukaryotic organisms

1. High throughput sequencing
2. Assembly into contigs
3. Search for similarity in databases using BLAST

RdRp

Systemic signal

Maintenance, amplification

”Degradative PCR”

DNA-virus (e.g. geminivirus)

overlapping ssRNAs

Replicating RNA virus

dsRNA (RNA virus)

Initiation

Rnase

siRNA

RISC nuclease complex

Targeted RNA degradation

Complementary ssRNA or dsRNA
Procedure: simple!

Extract RNA & run in 4% agarose gel

Cut and purify 20-30 nt band, prepare library

Send to sequencing provider

Bio-informatics
Deep sequencing of small RNAs from virus infected sweetpotato
Deep sequencing of small RNAs from virus infected sweetpotato
Deep sequencing of small RNAs from virus infected sweetpotato

- *Sweetpotato feathery mottle virus* (SPFMV; genus *Potyvirus*, family *Potyviridae*), *Sweetpotato chlorotic stunt virus* (SPCSV; genus *Crinivirus*; family *Closteroviridae*) and SPFMV+SPCSV infected sweetpotato leaf samples
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- Isolates not yet sequenced
- Short sequence assemblers may be able to assemble viral siRNAs?
Proof of concept

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*Note: Cov = coverage, Blastx = Translated Nucleotide Blast*
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<td>SPC CSV 2 (≤ 81)</td>
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<td>Badnavirus 38 (≤ 256)</td>
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<td>Mastrevirus 5 (≤ 210)</td>
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![Diagram of SPFMV and SPC CSV contigs](image1)

![Diagram of Sweetpotato pakakuy virus A & B](image2)

![Diagram of Sweetpotato mastrevirus?](image3)
Other plant species

Comparative sequencing of plant small RNAs
http://smallrna.udel.edu/
Other plant species

Comparative sequencing of plant small RNAs
http://smallrna.udel.edu/

* similarity to caulimoviridae
Other plant species

Cucurbita maxima (pumpkin)  
Fabaceae (Medicago, soybean)  
Populus (poplar)  
Gossypium (cotton)  
Citrus sinensis (orange)  
Arabidopsis  
Carica papaya (papaya)  
Silene latifolia  
Vitis vinifera (grape)  
Antirrhinum (snapdragon)

Petunia  
Tobacco  
Pepper  
Tomato  
Potato

Lactuca sativa (lettuce)  
Mimulus guttatus  
Vaccinium (blueberry)  
Phasmatobryanthemum (ice plant)  
Beta (beet)  
Eschscholtzia (California poppy)

Persea americana (avocado)  
Liriodendron (yellow poplar)

Zostera marina (sea grass)  
Zostera angulata (coastal worm)

Musa acuminata (banana)

rice  
Wheat  
Barley  
Brachypodium  
Switchgrass  
Festuca millet  
Maize  
Sorghum  
Miscanthus

Nuphar lutea (water lily)  
Amborella trichopoda

Picea abies (Norway spruce)  
Pinus radiata (Lobolly pine)  
Gnetum  
Ginkgo biloba  
Gycas rumphii (cycad)

Marsilea quadrifolia  
Anomochilus  
Psilotum  
Selaginella  
Lycopodium

Physcomitrella (moss)  
Marchantia (liverwort)  
Chara  
Heterosigma  
Acetabularia  
Chlamydomonas  
Volvox

Integrated badnaviruses

Comparative sequencing of plant small RNAs
http://smallrna.udel.edu/

similarity to caulimoviridae
Other plant species

Comparative sequencing of plant small RNAs
http://smallrna.udel.edu/

TVCV (integrated)

Integrated badnaviruses

* similarity to caulimoviridae
Beyond retroviruses
Beyond retroviruses

- Silene latifolia
- Physcomitrella (moss)
- New totivirus

- Similarity to Caulimoviridae
Beyond retroviruses

- new totivirus
- Papaya ring spot virus: 99.6% coverage

_similarity to Caulimoviridae_
Beyond retroviruses

- **new totivirus**
- **Papaya ring spot virus**: 99.6% coverage
- **Bell pepper endornavirus** (Endornavirus)

- **similarities to Caulimoviridae**
Beyond retroviruses

- new totivirus
- Papaya ring spot virus: 99.6% coverage
- Bell pepper endornavirus (Endornavirus)
- Lettuce big-vein associated virus (Varicosavirus)
- Mirafiore lettuce virus (Ophiovirus)

Similarity to Caulimoviridae
Beyond retroviruses

- new totivirus
- Papaya ring spot virus: 99.6% coverage
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- Mirafiore lettuce virus (Ophiovirus)
- Rice ragged stunt virus (Oryzavirus; Reoviridae)

* similarity to Caulimoviridae
Beyond retroviruses

- New totivirus
- Papaya ring spot virus: 99.6% coverage

White Campion
- New partitivirus
- New totivirus

Bell pepper endornavirus (Endornavirus)

Lettuce big-vein associated virus (Varicosavirus)

Mirafiore lettuce virus (Ophiovirus)

Rice ragged stunt virus (Oryzavirus; Reoviridae)

* similarity to Caulimoviridae
Beyond retroviruses

- **new totivirus**
- **Papaya ring spot virus**: 99.6% coverage
- **Bell pepper endornavirus** (**Endornavirus**)
- **Lettuce big-vein associated virus** (**Varicosavirus**)
- **Mirafiore lettuce virus** (**Ophiovirus**)
- **Rice ragged stunt virus** (**Oryzavirus; Reoviridae**)
- **Cucumber mosaic virus**
- **new Cytorhabdovirus**

- **similarly to Caulimoviridae**
Beyond retroviruses

- New totivirus
- Papaya ring spot virus: 99.6% coverage
- Rice ragged stunt virus (Oryzavirus; Reoviridae)
- Bell pepper endornavirus (Endornavirus)
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- Mirafiore lettuce virus (Ophiovirus)
- Cucumber mosaic virus
- New partitivirus
- New totivirus
- New Cytorhabdovirus
- Beet western yellows virus

- Similarity to Caulimoviridae
Tissue specificity?

- Cucurbita maxima (pumpkin)
- Fabaceae (Medicago, soybean)
- Malus (apple)
- Populus (poplar)
- Gossypium (cotton)
- Citrus sinensis (orange)
- Arabidopsis
- Carica papaya (papaya)
- Silene latifolia
- Vitis vinifera (grape)
- Antirrhinum (snapdragon)
- Petunia
- Tobacco
- Pepper
- Tomato
- Potato
- Lactuca sativa (lettuce)
- Mimulus guttatus
- Vaccinium (blueberry)
- Physooxybryanthemum (ice plant)
- Beta (beet)
- Eschscholtzia (California poppy)
- Persea americana (avocado)
- Linoleatrum (yellow poplar)
- Zostera marina (sea grass)
- Vitis vinifera (grape palm)
- Musa acuminata (banana)
- rice
- Wheat
- Barley
- Panicum
- Switchgrass
- Foxtail millet
- Maize
- Sorghum
- Miscanthus
- Nelumbo nucifera (water lily)
- Amborella trichopoda
- Picea abies (Norway spruce)
- Pinus sylvestris (Lobolly pine)
- Gnetum
- Ginkgo biloba
- Gycas rumphii (cycad)
- Marsilea quadrifolia
- Anigozanthos
- Psilotum
- Selaginella
- Lycopodium
- Physcomitrella (moss)
- Marchantia (liverwort)
- Chara
- Mesostigma
- Acetabularia
- Chlamydomonas
- Vorticella
Tissue specificity?

Silene latifolia

Physcomitrella (moss)

Leaves
Flowers
Boll fibres

✓ totivirus
Tissue specificity?

Silene latifolia (physcomitrella (moss))

Bell pepper endornavirus (Endornavirus)

Leaves

Flowers

Boll fibres

✓ totivirus

✓ Bell pepper endornavirus (Endornavirus)
Tissue specificity?

- **Silene latifolia** (moss)
  - Leaves
  - Flowers
  - Fruits

- **Bell pepper endornavirus (Endornavirus)**
  - Leaves
  - Flowers
  - Boll fibres

- **Cucumber mosaic virus**
  - Leaves
  - Flowers

- **Totivirus**
  - Leaves
  - Flowers
  - Boll fibres

- **Cucumber mosaic virus**
  - Roots
  - Leaves
  - Flowers

- **New Cytorhabdovirus**
  - Roots
  - Leaves
  - Flowers
Tissue specificity?

Leaves
Flowers ↓↓↓
Boll fibres ↓↓

✅ totivirus

Leaves
Flowers 

✅ Bell pepper endornavirus (Endornavirus)

Fruits

✅ Cucumber mosaic virus
✅ new Cytorhabdovirus

Roots
Leaves

✅ Beet western yellows virus

Draught stress ↑↑↑
Tissue specificity?

- **Silene latifolia** (rice, Physcomitrella (moss))
  - Leaves
  - Flowers
  - Fruits

- **Bell pepper endornavirus (Endornavirus)**
  - Leaves
  - Flowers
  - Boll fibres

- **Cucumber mosaic virus**
  - Leaves
  - Flowers
  - Fruits

- **Beet western yellows virus**
  - Roots
  - Leaves
  - Flowers

- **Partitivirus**
  - Leaves
  - Flowers
  - Smutted flowers

- **Totivirus**
  - Leaves
  - Flowers
  - Boll fibres

- **Chrysovirus**
  - Leaves
  - Flowers
  - Smutted flowers

- **New Cytorhabdovirus**
  - Leaves
  - Flowers

- **Beet western yellows virus**
  - Roots
  - Leaves
  - Draught stress
Better assembly with faster sample preparation methods: higher sensitivity
Small RNA sequencing and assembly: applications

- Rapid identification & sequencing of new virus (strains):
Small RNA sequencing and assembly: applications

- Rapid identification & sequencing of new virus (strains):
  - yambean: YBMV (Fuentes et al., 2012)
Small RNA sequencing and assembly: applications

- **Rapid identification & sequencing of new virus (strains):**
  - yambean: **YBMV** (Fuentes et al., 2012)
  - sweetpotato: **SPFMV** (Untiveros et al., 2010) & **SPCSV** (Cuellar et al., 2011), **SPC6V** (De Souza et al., 2013), **SPC3V**, **SPVZ**, alpha satellites
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  - potato: APLV & APMMV (Kreuze et al., 2013), vitivirus, crinivirus, nepovirus, ophiovirus, torrado-like
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- Generic virus indexing method
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- Generic virus indexing method
- Using for mapping and monitoring virus distribution & variability, e.g. pan-African sweetpotato virome
Small RNA sequencing and assembly: applications

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  - arracacha: enamovirus, ST-9 like... and more
- Generic virus indexing method
- Using for mapping and monitoring virus distribution & variability, e.g. pan-African sweetpotato virome
Pathogen variability studies: PVY & PVX
Going to scale: the pan African sweetpotato virome

- ~2000 samples and bioinformatics pipeline for virus identification using siRNA assembly and genome subtraction

1. **raw Illumina sRNA reads**
2. **remove barcode, adaptor, low quality and low complexity sequences**
3. **cleaned sRNA reads**
4. **align to draft sweetpotato genome and EST sequences**
5. **unmapped reads align to other plant genomes**
6. **sweetpotato reads de novo assembly**
7. **virus genome contigs blast against nr and nt database, compare against Interpro and pfam**
8. **distribution of sRNA reads on virus genome contigs**
9. **align sRNA reads back to virus genome contigs**
10. **design primers for PCR and Sanger sequencing**
11. **Fill gaps and detect virus variants**
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<th>Region</th>
<th>District</th>
<th>Locality</th>
<th>Field number</th>
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<th>Latitude</th>
<th>Altitude (m)</th>
<th>Crop age (months)</th>
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<th>Photo number field</th>
<th>Photo number plant</th>
<th>Photo number leaf</th>
<th>Intercrop</th>
<th>Cultivar/species</th>
<th>Notes</th>
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**Field photo**

**Sampled leaf photo**

**Plant photo**
### Recorded data

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**Field photo**

**Standard color chart for image correction**

**Sampled leaf photo**

**Plant photo**

**Bar-coded**

**Size measure**
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Virus variation

[Diagram showing the variation of different virus strains, including SPVG, SPV2, SPVZ, SPVC, SPFMV, and SPLV. Each strain is represented with a branch and a percentage value indicating the confidence level of the branching.]

SPMMV-Uganda

SPMMV-Mozambique
<table>
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<th>sample #</th>
<th>region</th>
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Cotton leaf curl Gezira alphasatellite - Burkina Faso

’Nodavirales superfamily’
What to do with this?

- Tombusviridae
- Umbravirus
- ST9 like RNA virus
- gp2 Marine RNA virus SOG
- Betatetravirus
- Chronic bee paralysis virus
- RdRp GAF 175
- RdRp protein partial uncultured virus
- Lake Sinai virus
- RdRp Sclerophthora macrospora virus A
- Plasmopara halstedii virus
- Nodaviridae
What to do with this?
Small RNA sequencing and assembly: lessons learnt

- All types of genomes (dsRNA, ssRNA, dsDNA, ssDNA, RT)
- Complete genomes can be assembled
- Sensitive: low titer symptomless infection & ELISA negative plants
- New viruses: no prior knowledge required
- Applicable to plants, fungi & animals
- Not all viruses produce abundant siRNAs
- Frequent mixed infection of field samples & defective RNA/DNA can make assembly difficult
- Risk of contamination between samples
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- A. Villamil, M. Guzmán
- Maya Ravnikar & Denis Kutnjak