Netherlands Food and Consumer
Product Safety Authority
Ministry of Economic Affairs

Comparison of NGS pipelines and traditional diagnostics in annual *Daucus carota* surveys

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Overview presentation

- Phytosanitary survey in carrot
- Traditional diagnostics
- Next Generation Sequencing approach
  - Selection of reference genes
  - Reference based detection
  - *De novo* and blast based detection
- Comparison of costs and hands-on time

Phytosanitary survey *Daucus carota* (carrot)

- Annual survey in carrot since 2011

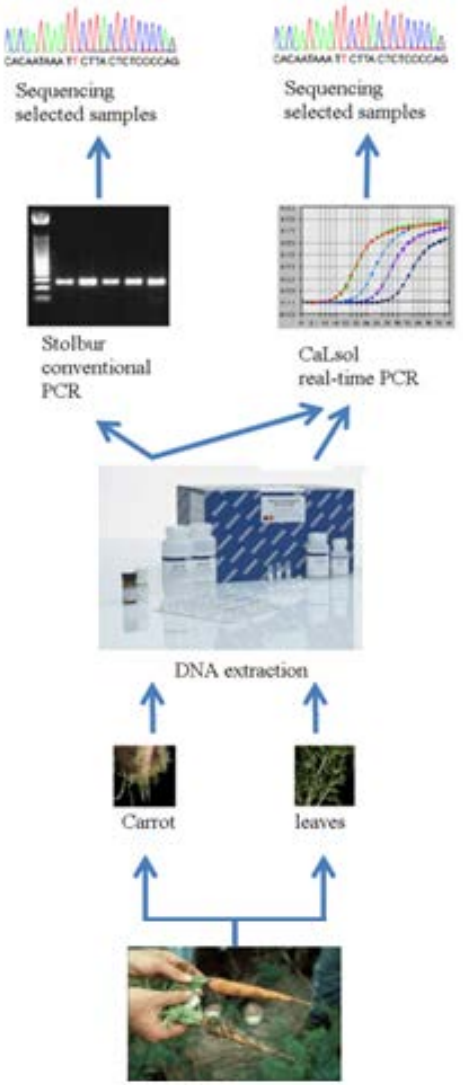
- Presence of
  - *Ca*. Liberibacter solanacearum (CaLsol) (EPPO A1)
  - *Ca*. Phytoplasma solani (CaPsol) (EU II/A2)

Symptomatic material

- ~130 inspections resulting in ~30 samples
  - Presently, both pests have not been detected
- Sampling of symptomatic field-grown carrots
  - Discolored leaves (red, yellow)
  - Stunted growth
  - Formation of side roots
- Symptoms not specific to both pests
  - *Ca* P. asteris suspected causal agent in majority of cases

Diagnostic testing scheme

- Two subsamples (leaves & carrot)
- Detection by:
  - Conventional PCR CaPsol (leaves)
  - Real-time CaLsol (leaves & carrot)
- Verification for selected samples
  - targeted PCR Sanger sequencing

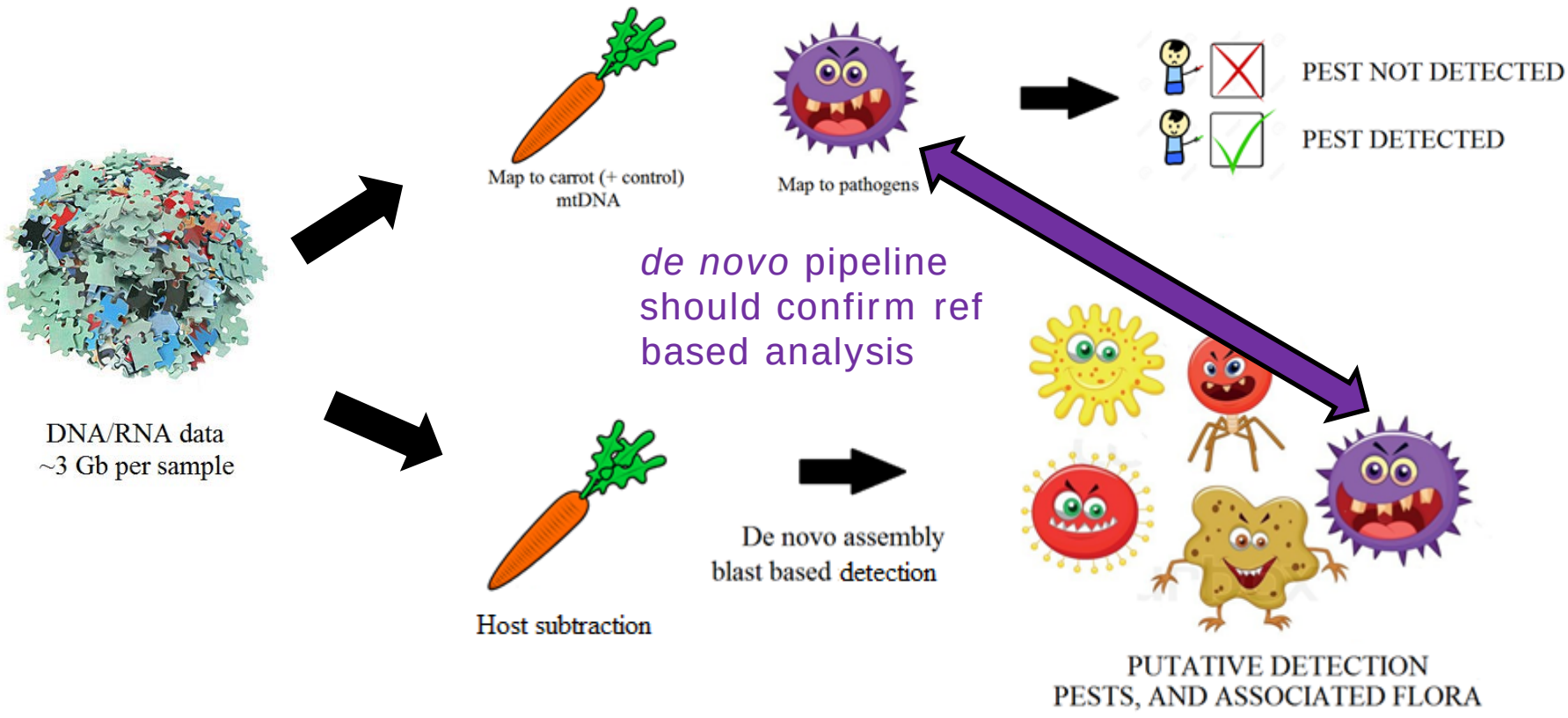


The iterative use of test methods:
1. is time consuming
2. requires lots of hands-on time
3. is therefore costly

Next Generation Sequencing (NGS); *an alternative?*

- Why using the *Daucus carota* survey?
  - Specific scope: 2 pests in symptomatic material (analytical sensitivity)
  - Availability of reference sequences (analytical specificity)
  - Survey shared by multiple disciplines
  - “Long” turn-over time allowed

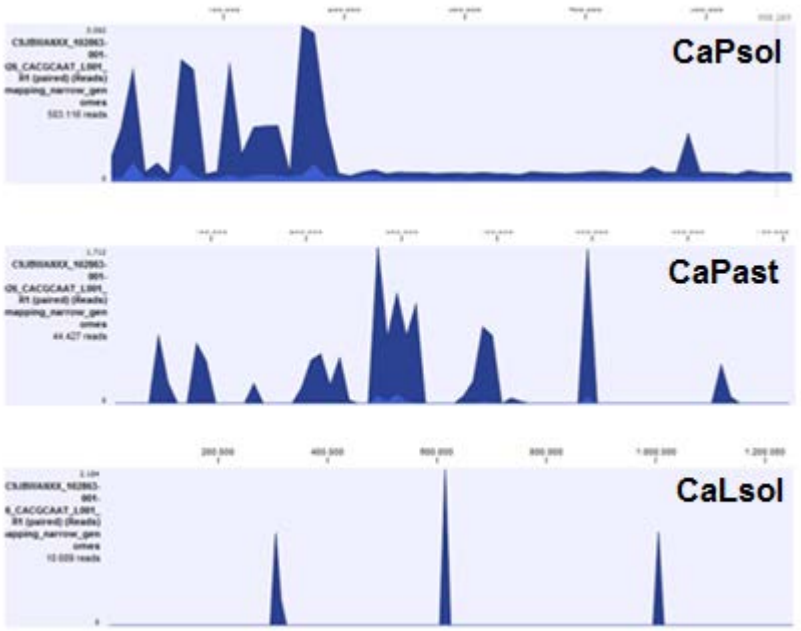
Analysis pipelines – reference vs. *de novo*



Defining suitable reference sequences

Entire genome not suitable for detection

- CaPsol, CaLsol and CaPast genomes share homology (non-specific mapping)
- Regions with variable resolution (non-species level resolution)
- Determining cut-offs for detection using the entire genome is not possible

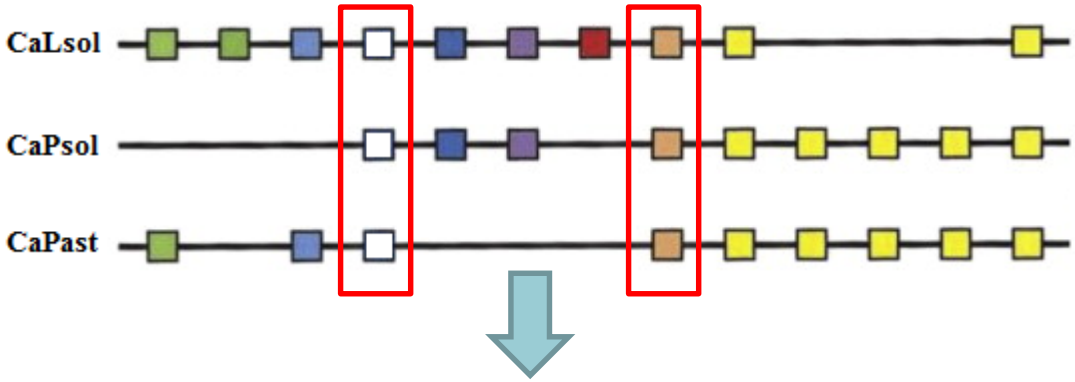


CaPsol sequence data mapped to CaPsol, CaPast and CaLsol

Selection of reference genes

Suitable reference genes are:

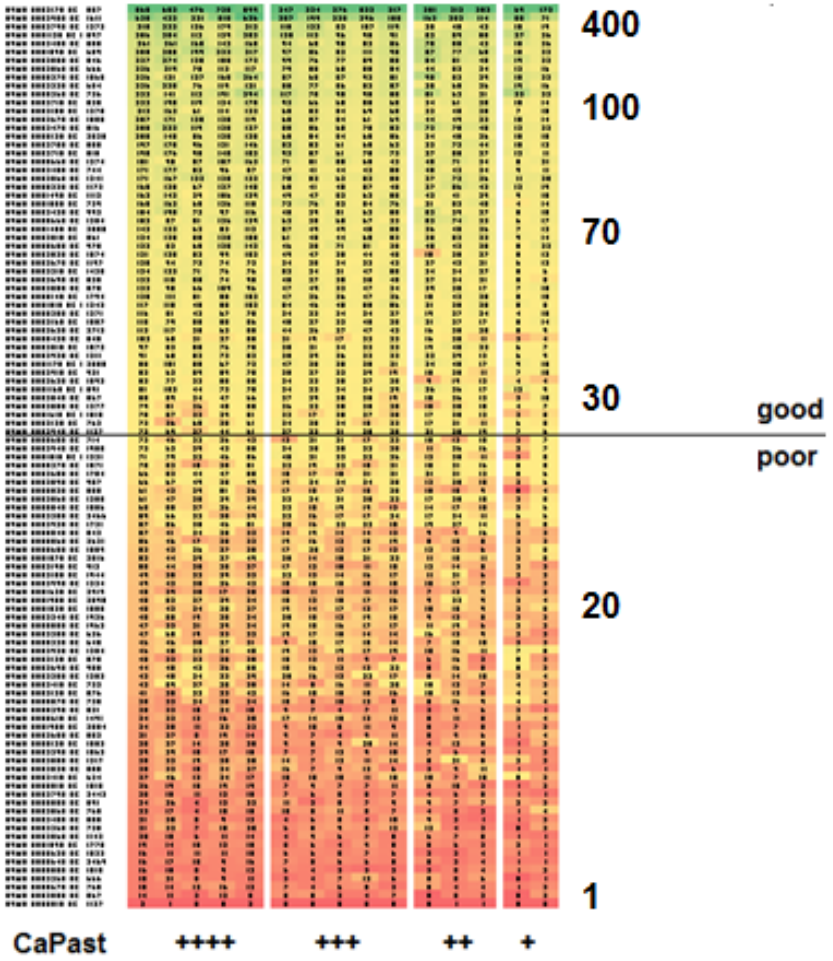
- Single copy orthologs (SCO)
  - Even coverage expected
  - Can be compared over species
- SCO with species level resolution
- SCO >500 nt for reliable mapping



107 reference genes per species

Not all 107 species specific SCOs can be used for RNAseq pipeline

- Selected reference genes for DNA pipeline are not equally transcribed
- Transcription level per SCO is conserved over samples
  - Highly expressed gene in sample 1 = highly expressed gene in sample 2
- 51 species specific SCOs with at least >5x average coverage in individual samples were selected for RNAseq pipeline



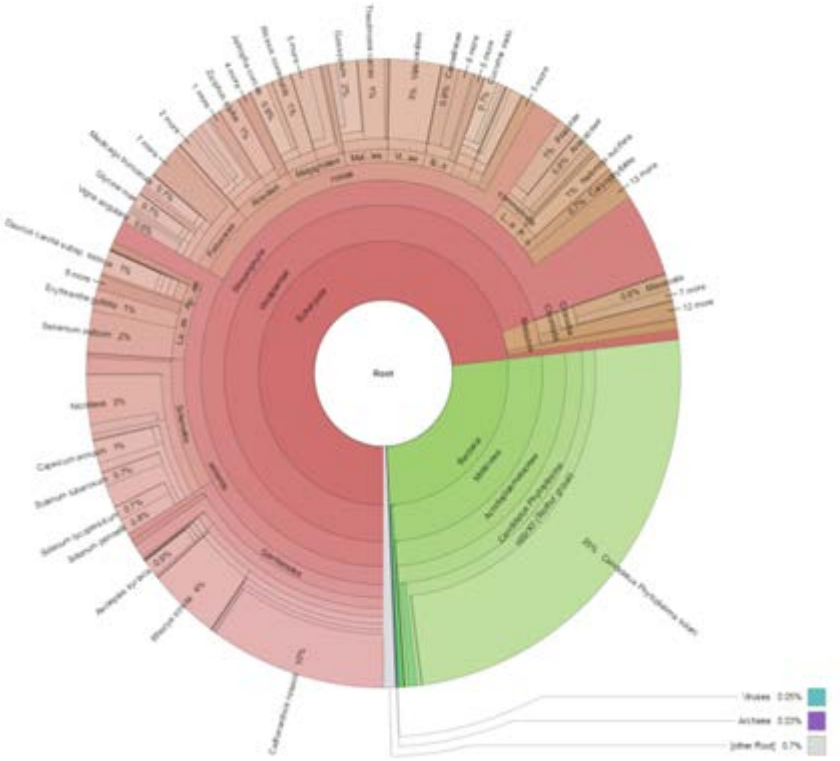
Reference assembly results – DNA and RNA

LIMS number	Traditional diagnostics		DNA NextSeq			RNA NextSeq		
	CaLsol/CaPsol detected	Suspected causal agent	CaLsol	CaPsol	CaPast	CaLsol	CaPsol	CaPast
5727954	NO	putative CaPast	NO	NO	+	NO	NO	+
5727962	NO	CaPast	NO	NO	+	NO	NO	+
5727970	NO	CaPast	NO	NO	+	NO	NO	+
5727989	NO	putative CaPast	NO	NO	+	NO	NO	+
5727997	NO	putative CaPast	NO	NO	+	NO	NO	+
5728009	NO	putative CaPast	NO	NO	+	NO	NO	+
5728228	NO	putative CaPast	NO	NO	+	NO	NO	+
5728244	NO	CaPast	NO	NO	+	NO	NO	+
5728367	NO	unknown	NO	NO	NO	NO	NO	NO
5728375	NO	CaPast	NO	NO	+	NO	NO	+
5728391	NO	CaPast	NO	NO	+	NO	NO	+
5728420	NO	putative CaPast	NO	NO	+	NO	NO	+
5728439	NO	putative CaPast	NO	NO	+	NO	NO	+
5728447	NO	unknown	NO	NO	NO	NO	NO	NO
5728455	NO	putative CaPast	NO	NO	+	NO	NO	+
5728463	NO	unknown	NO	NO	NO	NO	NO	NO
5728471	NO	CaPast	NO	NO	+	NO	NO	+
5974066	NO	putative CaPast	NO	NO	+	NO	NO	+
5974074	NO	unknown	NO	NO	NO	NO	NO	NO
6792299	NO	unknown	NO	NO	NO	NO	NO	NO

- Identical qualitative results were obtained from the DNA and RNA detection pipelines
- Pipeline output could easily be interpreted

de novo assembly + blast-based detection

- Beyond the initial scope of the survey
- When the usual suspects cannot be detected, are there other possible causal agents that could explain the symptoms observed?
- Blast-based detection: indicative and use with caution!
- Interactive visualisation tool: Krona



Possible candidates for observed symptoms

- Carrot viruses detected:
  - Carrot torrado virus 1
  - Carrot cryptic virus
  - Carrot mottle virus
  - Carrot red leaf luteovirus associated RNA
  - Carrot read leaf virus

- Carrot read leaf virus and Carrot mottle virus were detected in all CaPast negative samples (#5) and possibly causing the observed symptoms of Carrot motley dwarf (CMD)

What about the money?

- Direct costs are higher per sample
- Hands-on time is greatly reduced per sample
  - Traditional: 57 min/sample
  - NGS: 21 min/sample
- Net extra costs per sample: €89
  - Saving in hands-on time
  - Re-usable datasets
  - Possibilities for detection beyond initial scope of survey

Traditional diagnostics	costs per sample
DNA extraction (Qiagen, plant mini kit)	€ 8,00
Bio-X-ACT short mix reactions	€ 2,00
TaqMan universal Master Mix	€ 9,00
Qiaquick PCR Purification Kit	€ 2,00
BigDye terminator kit v1.1	€ 21,00
DyeEx kit	€ 6,00
Pop7 polymer	€ 1,00
Capillary	€ 2,00
Total	€ 51,00

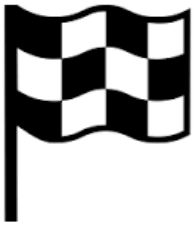
NGS approach (DNA)	costs per sample
DNA extraction (Qiagen, plant mini kit)	€ 4,00
NGS NextSeq, DNA, PE150, 4.5 Gb data	€ 320,00
Total	€ 324,00

Conclusions

- We created a robust and reliable detection pipeline for CaPsol, CaLsol and CaPast detection in symptomatic carrot material

Costs

NGS: +€89 per sample



Turnover time

NGS: similar (~3 weeks)

Hands-on time

NGS: less than half (21 vs 57 minutes)

Results

Data can be used for:

1. specific detection of survey targets
2. detection beyond initial scope of survey
3. Additional analyses (e.g. track & trace)

Future work

- Create scripts for automated generation of result forms including results and conclusions for the different analyses
  - User-friendly, interactive, but stand-alone and write protected (QA)
  - In close collaboration with specialists from different disciplines

- Determine performance criteria following PM7/98(2) for the analysis pipelines and compare those to traditional tests

- Increase computational power and storage for NGS data

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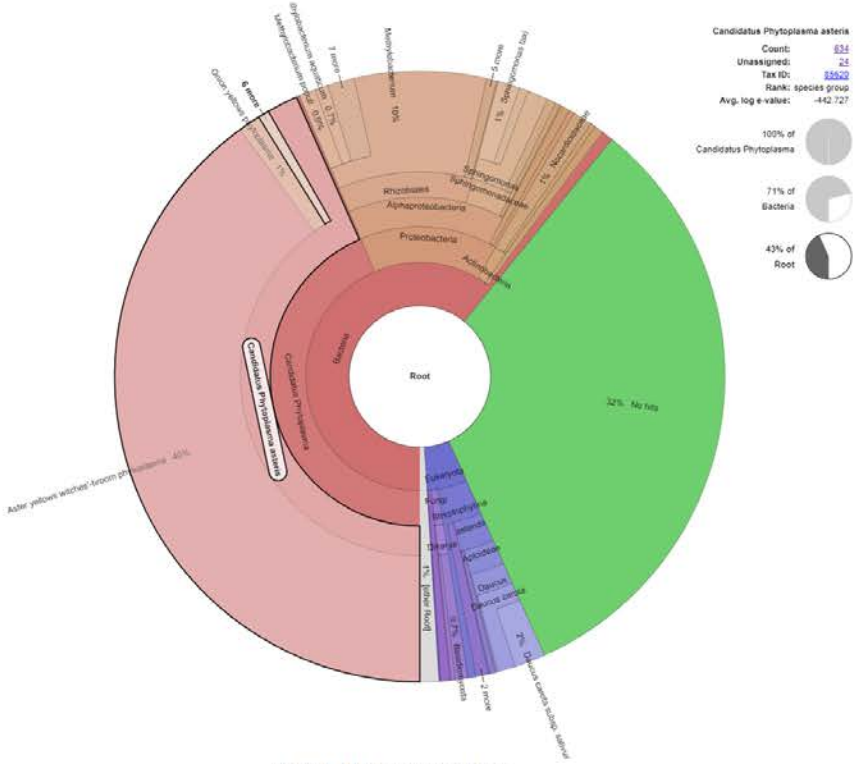
Thank you for your attention

RNAseq is not properly mapped to the host

***de Novo* assembly and blast-based detection**

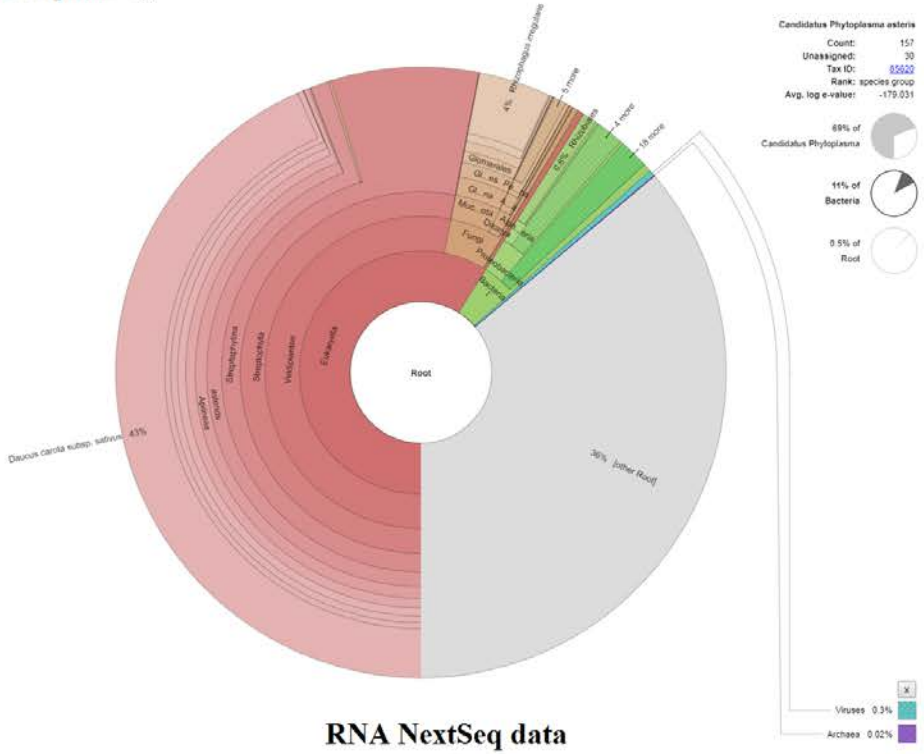
Sample_01 (CaPast positive)



DNA NextSeq data

1463 elements

585 CaPast hits **(40% of total)**



RNA NextSeq data

30,080 elements

157 CaPast hits **(0.5% of total)**

major groups: plant (53%)
unknown (36%)