In A Different Class?

Whole genome classification of bacterial potato pathogens

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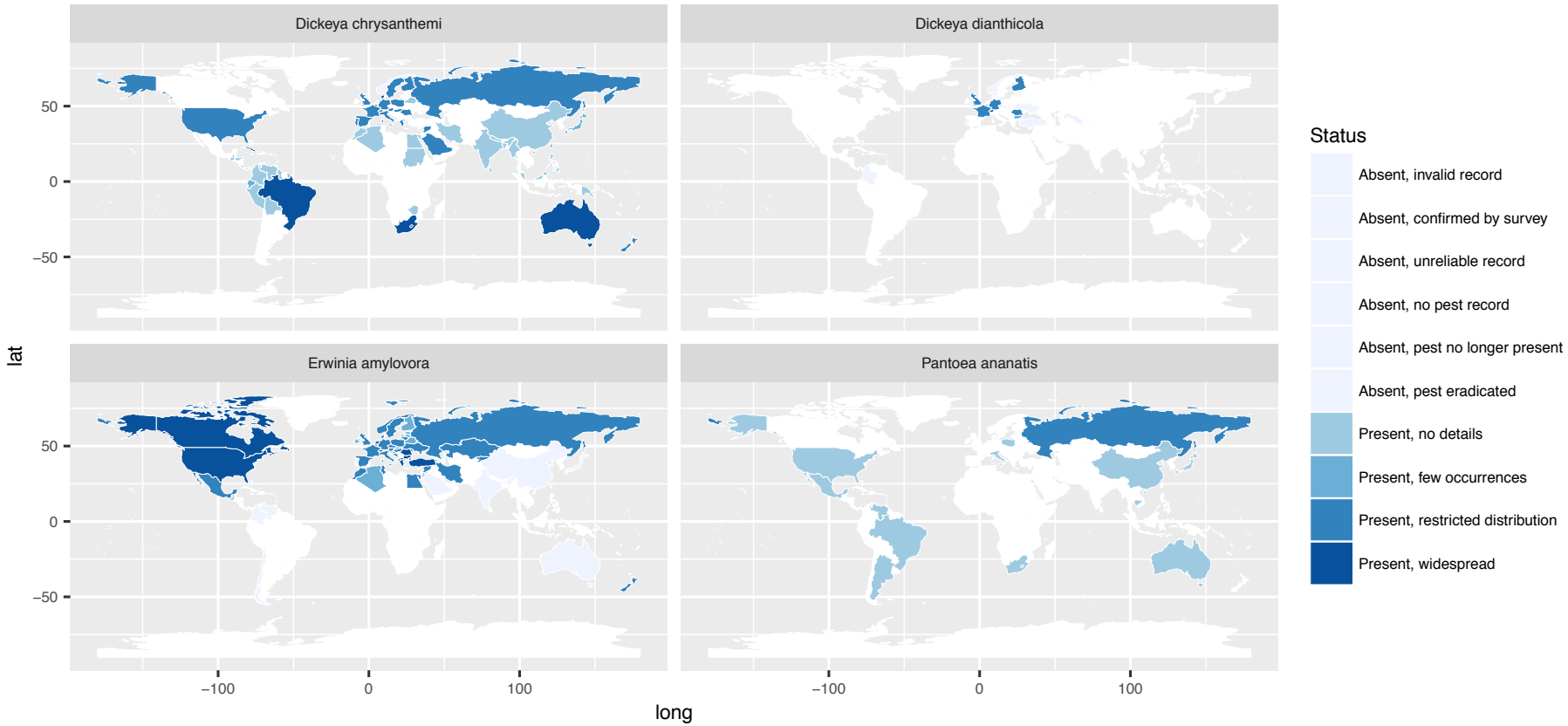
Plant-pathogenic Enterobacteria [a]

[a] Toth *et al.* (2006) *Annu. Rev. Phytopath.* doi:10.1146/annurev.phyto.44.070505.143444

- *Erwinia*, *Dickeya*, *Pantoea* and *Pectobacterium* spp.
- Plant Cell Wall Degrading Enzymes (PCWDEs)

Global threat [a]

[a] EPPO Global Database



A Tangled Taxonomy [a] [b]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h
[b] Williams *et al.* (2010) *J. Bact.* doi:10.1128/JB.01480-09

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Enterobacterial taxonomy difficult to resolve, in general
Soft rot enterobacteria (SRE): *Pectobacterium* and *Dickeya*

Historical classification mostly polyphasic/phenotypic

- SRE originally *Erwinia* spp., now three distinct genera (*Dickeya*, *Pectobacterium*, *Erwinia*)
- *Pectobacterium* spp. used to be *E. carotovora* (and *E. chrysanthemi*)
- *Dickeya* spp. used to be *P. chrysanthemi*
- Not suitable for facultative bacteria?

Binomial nomenclature not designed for large amounts of genome data, or organised metadata curation

Tangled Taxonomy of SRE[a]

[a] Czajkowski *et al.* (2015) *Ann. Appl. Biol.* doi:10.1111/aab.12166

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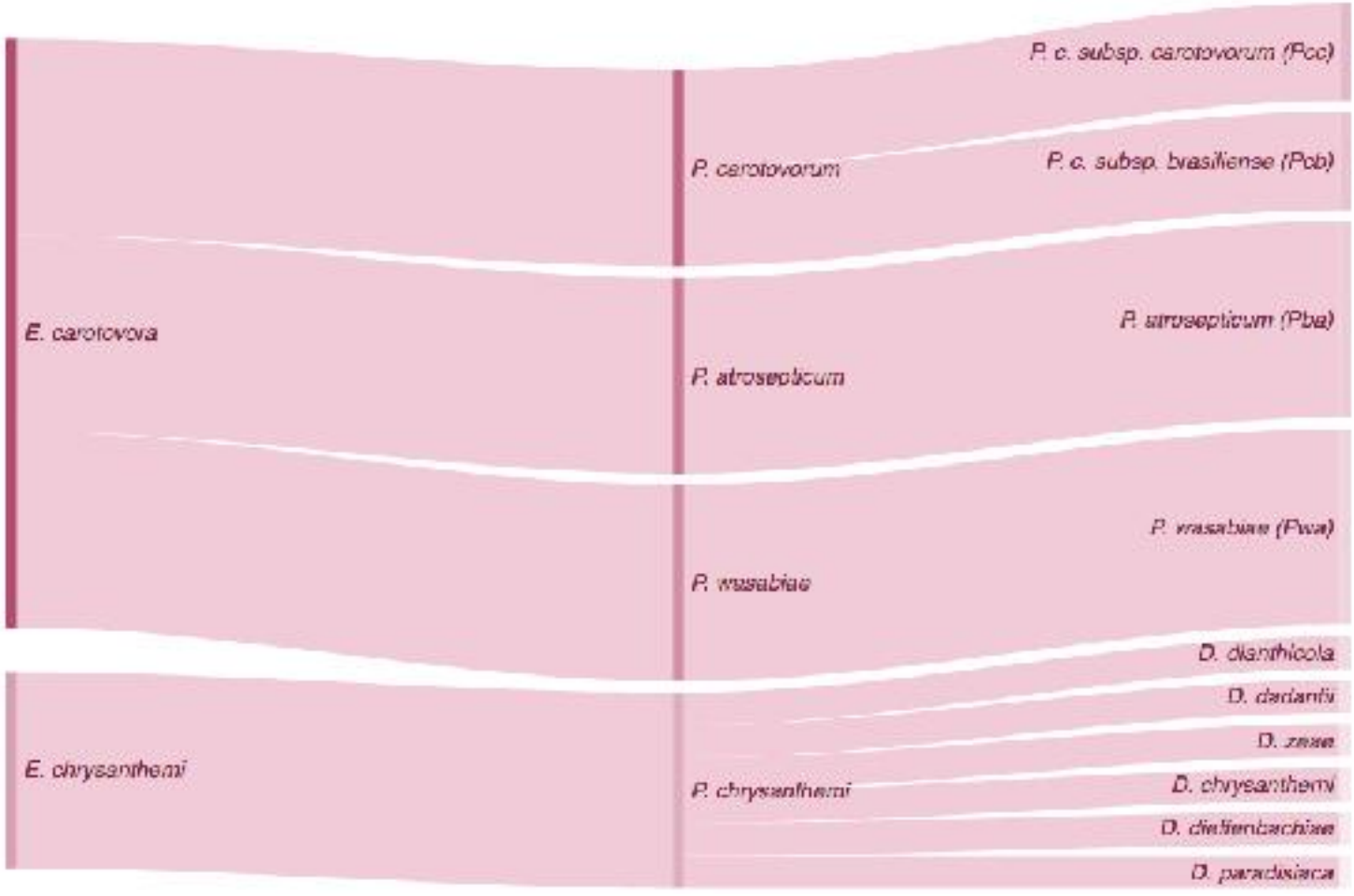
Old names hold over in the literature, collections, etc.

Name discontinuities affect analysis, databases



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Dickeya spp. moving across Europe [a] [b]

[a] Toth *et al.* (2011) *Plant Path.* doi:10.1111/j.1365-3059.2011.02427.x

[b] Parkinson *et al.* (2015) *Eur. J. Plant Path.* doi:10.1007/s10658-014-0523-5

D. dianthicola is established across Europe

D. solani is an emerging, encroaching threat

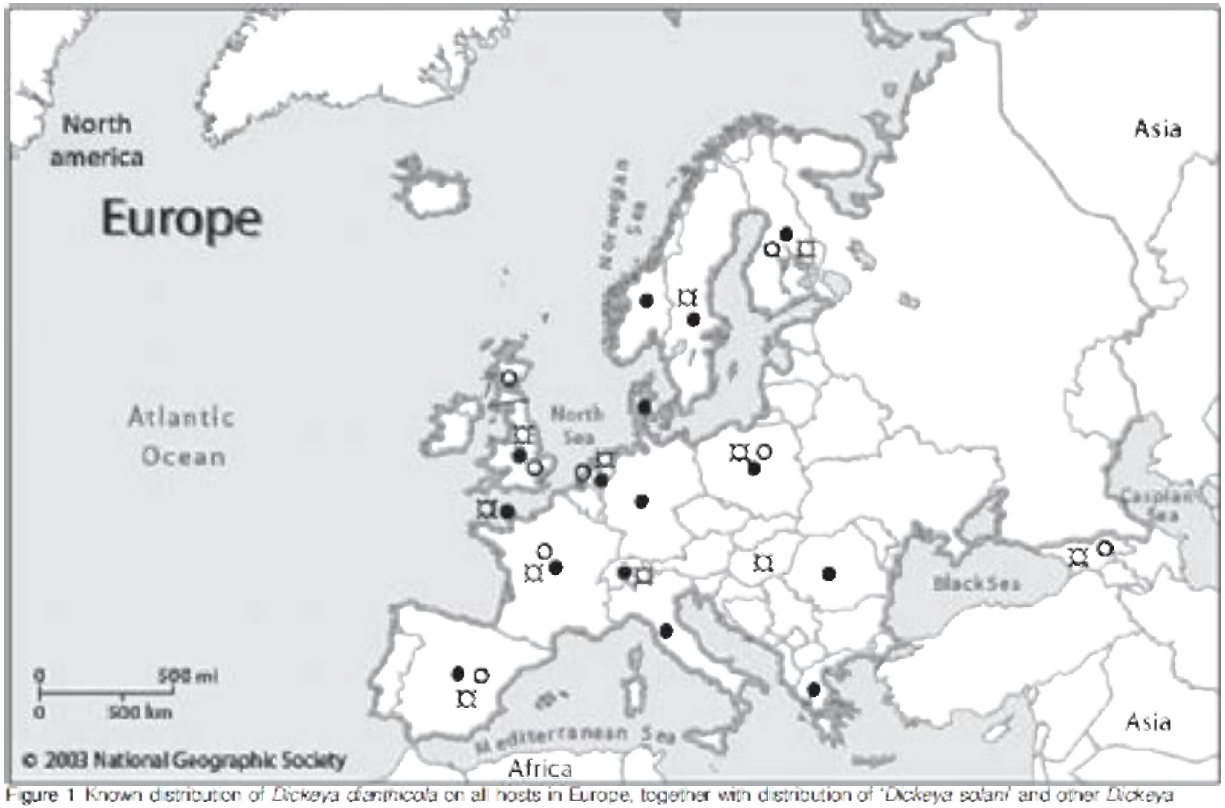

Figure 1 Known distribution of *Dickeya dianthicola* on all hosts in Europe, together with distribution of '*Dickeya solani*' and other *Dickeya* species on potato in Europe. Distribution of *D. dianthicola* on all hosts (updated from CAB International, 2006) (•); *Dickeya* spp. on potato (▫); '*D. solani*' on potato (○).

Legislation [a]

[a] Pritchard *et al.* (2015) *Anal. Methods* doi:10.1039/c5ay02550h

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European and Mediterranean Plant Protection Organisation (EPPO)

Member states should regulate *D. dianthicola* and *E. amylovora* as quarantine pests (A2 list)

Seed Potatoes (Scotland) Amendment Regulations (2010)

Zero tolerance policy for all *Dickeya* spp. on potatoes in Scotland to ensure production of 'clean' (disease-free) seed potato production for export

EUPHRESCO consortium

Euphresco : Control and epidemiology across Europe

Legislation by taxonomy [a]

[a] Pritchard *et al.* (2015) *Anal. Methods* doi:10.1039/c5ay02550h

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"Easy" to incorporate binomial nomenclature into legislation[1]

Assumption: taxonomy can be determined precisely

Taxonomy is a human-imposed hierarchical classification that truly reflects nature

Assumption: taxonomy is a proxy for disease risk

Sharing a common ancestor with another pathogen is the primary factor that influences virulence

[1] (easy for policy-makers to understand)

Issues with legislating by taxonomy [a]

[a] Pritchard *et al.* (2015) *Anal. Methods* doi:10.1039/c5ay02550h

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Is current bacterial taxonomy objective and correct?

- Taxonomy is 'vertical', but pathogenicity may be 'laterally' transferred (plasmid/transposon-borne, etc.) [2]
- Is a species concept even relevant for bacteria?

Mapping from taxonomy to phenotype is not one-to-one [3]

- Testing for disease phenotypes not exhaustive (facultative pathogens)
- Relationship between genome and disease/risk not fully understood

[2] Toth *et al.* (2006) *Ann. Rev. Phyto.* doi:10.1146/annurev.phyto.44.070505.143444

[3] Deans *et al.* (2015) *PLoS Biol.* doi:10.1371/journal.pbio.1002033

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Dickeya qPCR diagnostics [a] [b] [c]

[a] Pritchard *et al.* (2013) *Plant Path.* doi:10.1111/j.1365-3059.2012.02678.x
[b] Pritchard *et al.* (2013) *Genome Ann.* doi:10.1128/genomeA.00087-12
[c] Pritchard *et al.* (2013) *Genome Ann.* doi:10.1128/genomeA.00978-13

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To legislate on or quarantine contaminated materials, one has to be able to identify and discriminate the pathogen

- Having sequenced 25 *Dickeya* isolates, we were approached to develop diagnostics at the species/isolate level
- qPCR is cheaper, quicker and easier than bacterial genome sequencing (for now, anyway...) [4]
- No qPCR primers existed to distinguish among *Dickeya* spp.

[4] Czajkowski *et al.* (2015) *Ann. Appl. Biol.* doi:10.1111/aab.12166

qPCR Primer Design [a]

[a] Pritchard *et al.* (2012) *PLoS One* doi:10.1371/journal.pone.0034498

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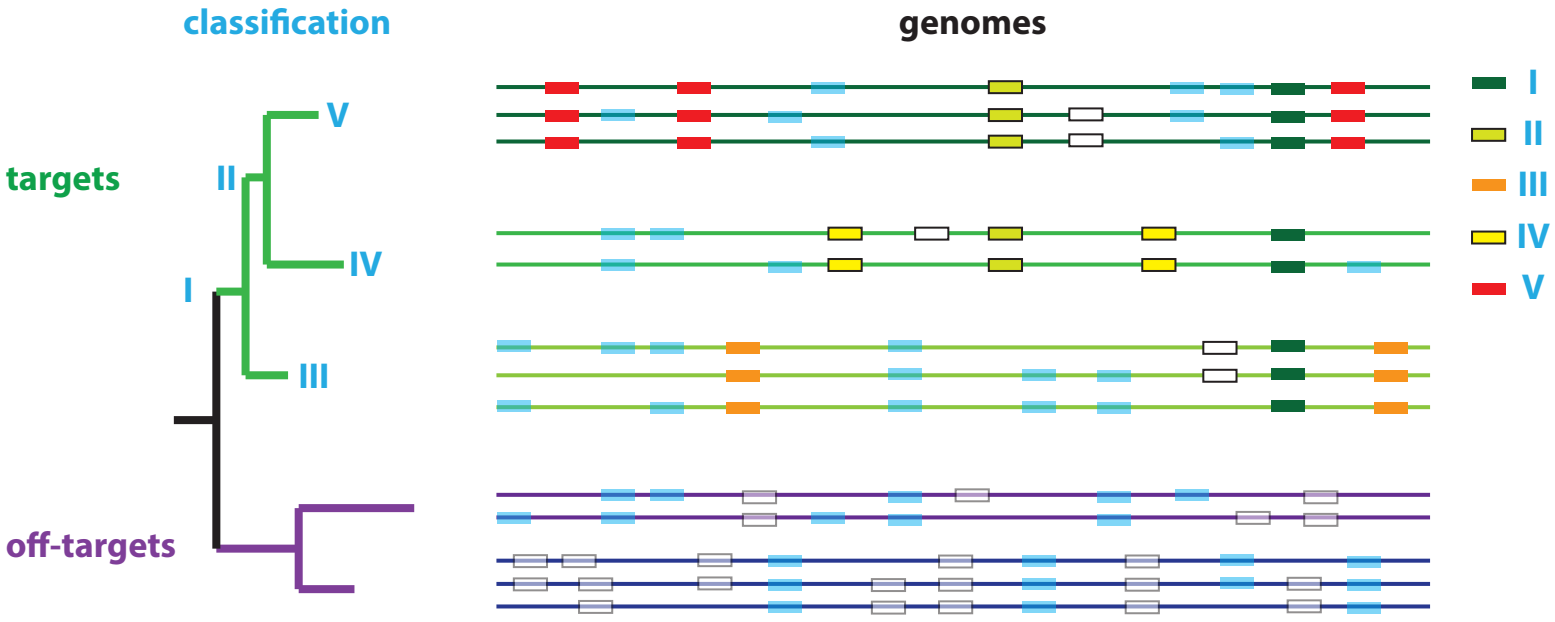
1. Bulk predict primer sets on all chromosomes (`Primer3`)
2. Predict cross-amplification *in silico* (`primersearch`)
3. Evaluate *in vitro* against panel of previously "unseen" isolates of known class and report performance metrics



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Classification is a problem! [a]

[a] Pritchard *et al.* (2013) *Plant Path.* doi:10.1111/j.1365-3059.2012.02678.x

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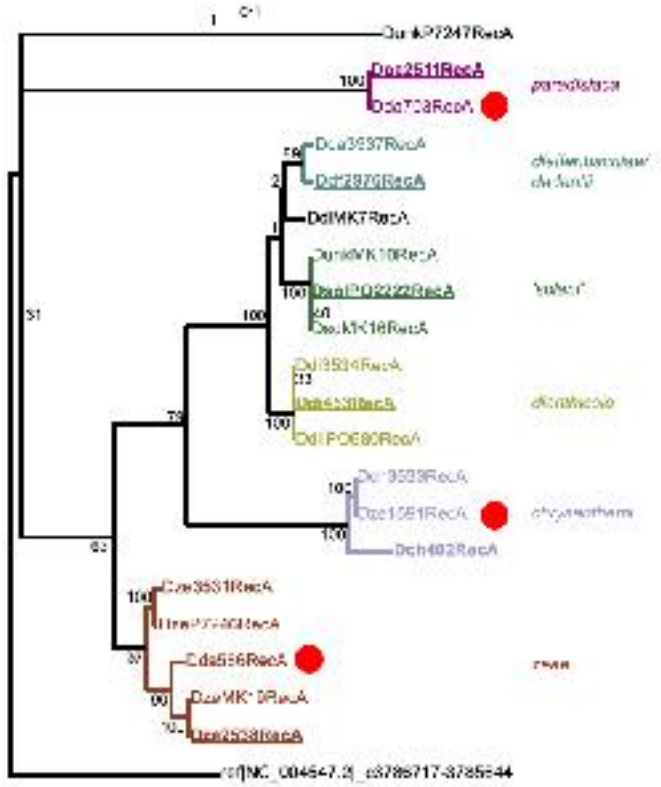
First qPCR design gave no diagnostic primers for several *Dickeya*!



[5]

Misassigned species in GenBank made 'training' impossible.

[5] ML tree, *recA*

Consequences of misclassification [a] [b]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h
[b] Varghese *et al.* (2015) *Nucl. Acids Res.* doi:10.1093/nar/gkv657

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Real-world consequences

- False positives (type I errors):
  clean samples rejected: economic cost
  farms quarantined/close: economic/societal cost
- False negatives (type II errors):
  (irreversible) introduction of infectious material
  potential for novel host jumps and spread

"Gold-standard", correctly classified training and test sets essential to estimate classifier error rates.

MiSI: 18% of NCBI bacterial genomes misclassified at species level

Accurate classification is essential!

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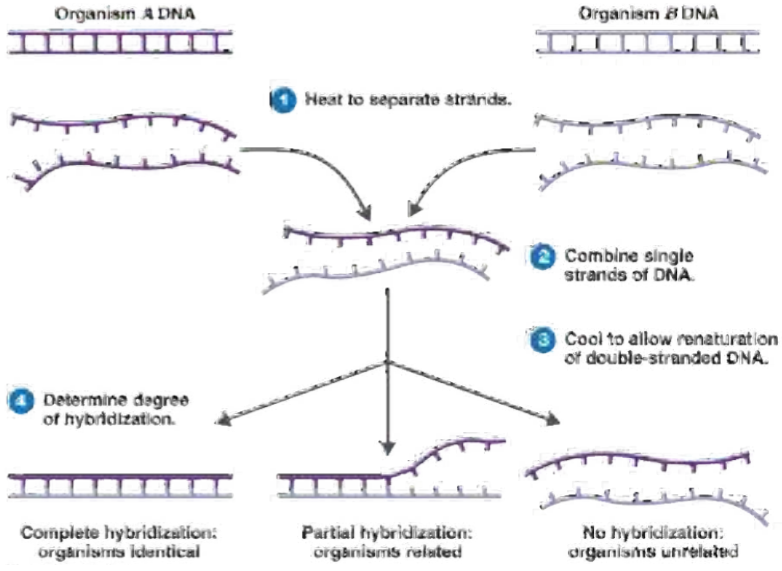
DNA-DNA hybridisation [a] [b]

[a] Morello-Mora and Amann (2001) *FEMS Micro. Rev.* doi:10.1016/S0168-6445(00)00040-1
[b] Chan *et al* (2012) *BMC Microbiol.* doi:10.1186/1471-2180-12-302

- "Gold Standard" for prokaryotic taxonomy, since 1960s. "70% identity ≈ same species."
- Denature DNA from two organisms.
- Allow to anneal. Reassociation ≈ similarity, measured as ΔT of denaturation curves.



Proxy for sequence similarity - replace with genome analysis?

Average Nucleotide Identity (ANIm) [a]

[a] Richter and Rossello-Mora (2009) *Proc. Natl. Acad. Sci. USA* doi:10.1073/pnas.0906412106

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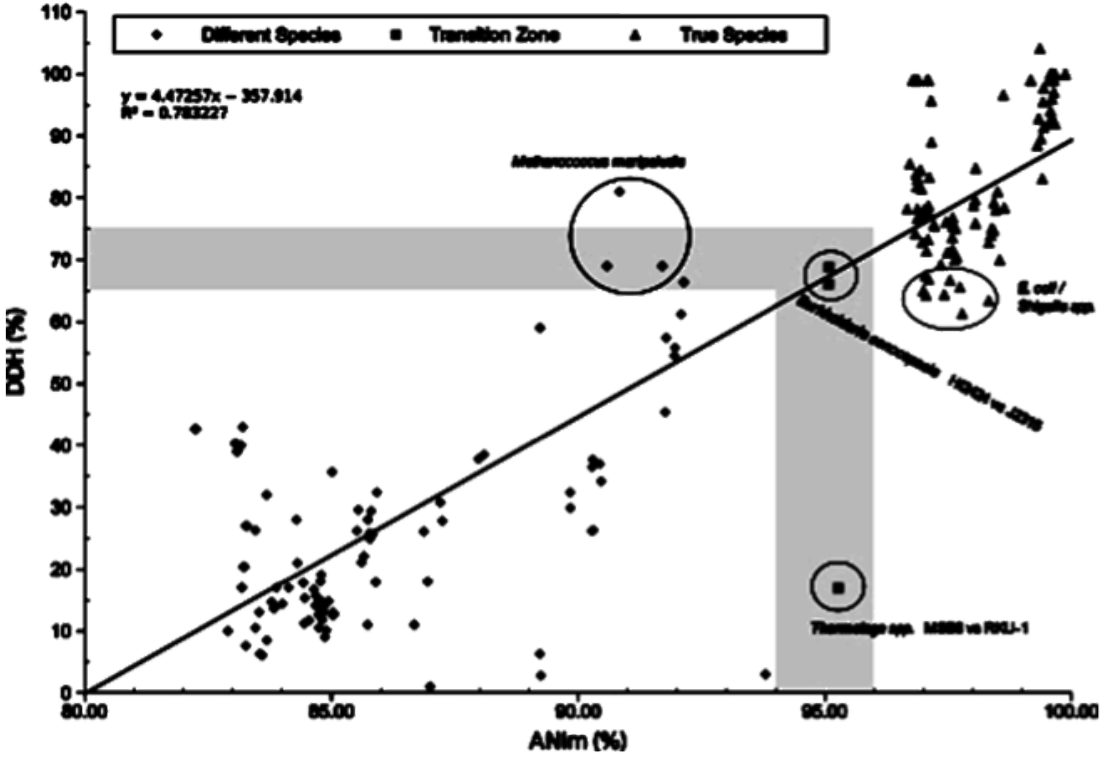
1. Align genomes (MUMmer)
2. **ANIm**: Mean % identity of all homologous region matches

- DDH:ANIm "linear"
- **70%ID ≈ 95%ANIm**



ANIm

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ANIm is. . .

- straightforward to apply to genomes
- average identity of all ‘homologous’ regions
- not dependent on dataset composition (unlike hierarchical clustering)
- (just) another pairwise distance measure
- approximate limiting case of MLST/MLSA/multigene comparisons

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pyani software [a]

[a] http://widdowquinn.github.io/pyani

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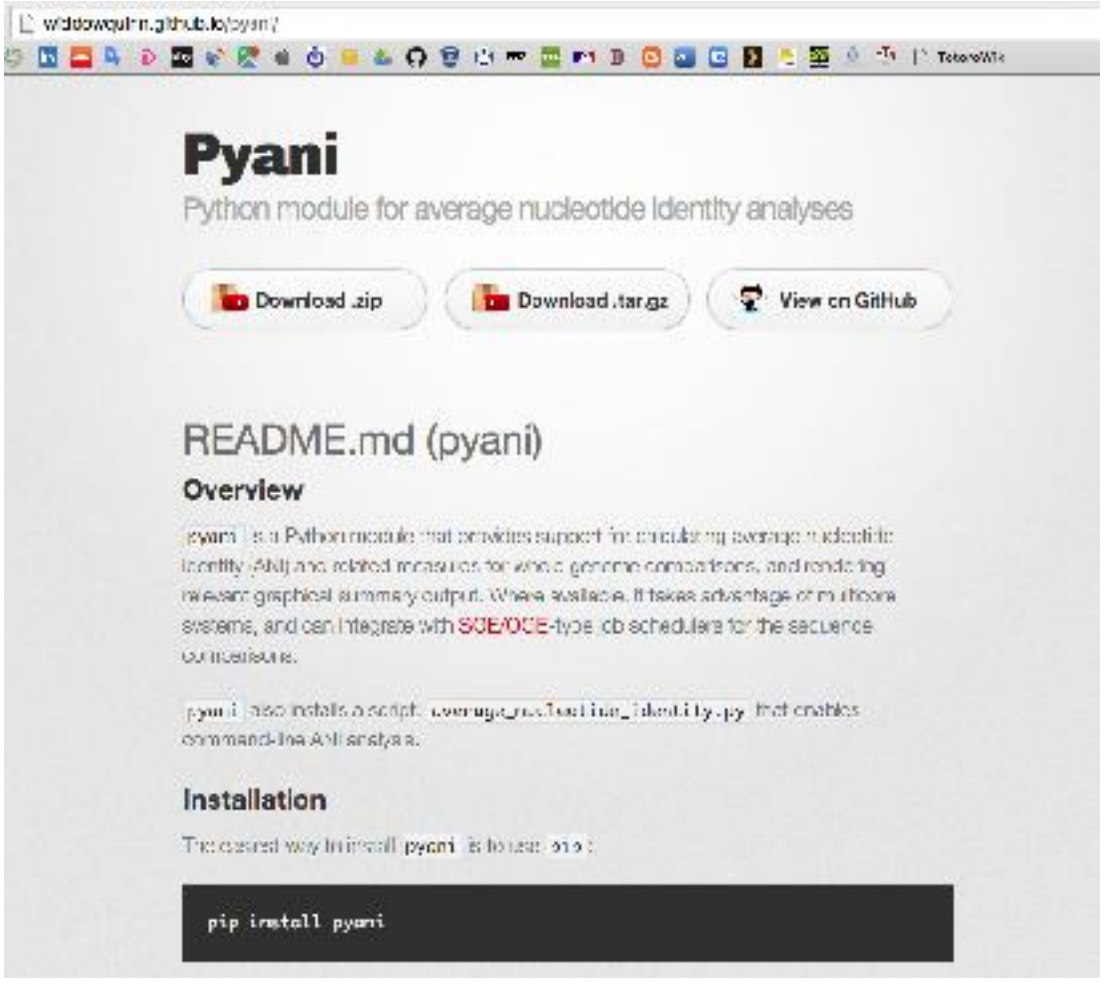
- Python ANI module and script
- Active development
- Parallelises on clusters
- Preprint coming soon (simulated genomes; lots of bacterial taxonomy!)



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34 Dickeya spp. ANIm [a]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h

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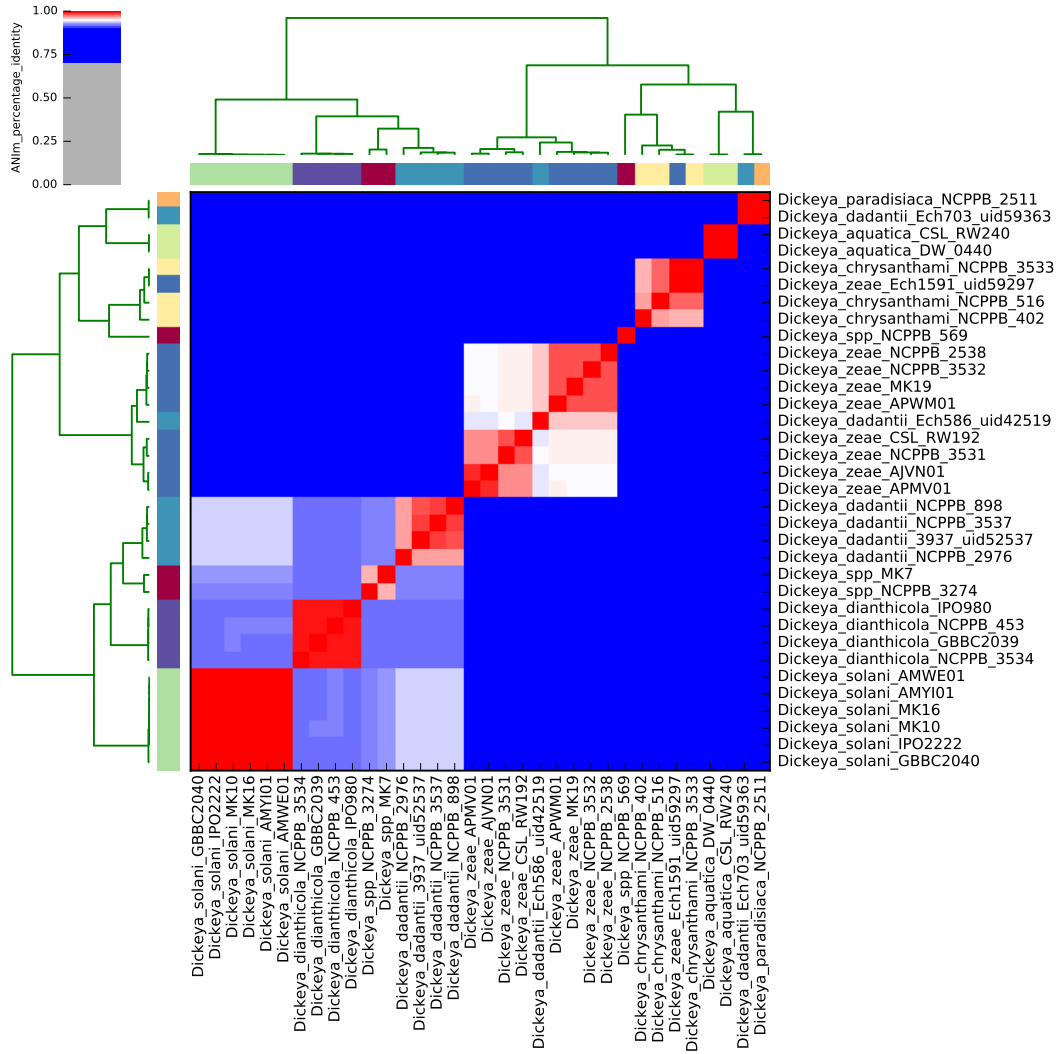
- Nine species-level groups (two novel)
- Correctly places three species misidentified in GenBank



55 Pectobacterium spp. ANIm [a]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h

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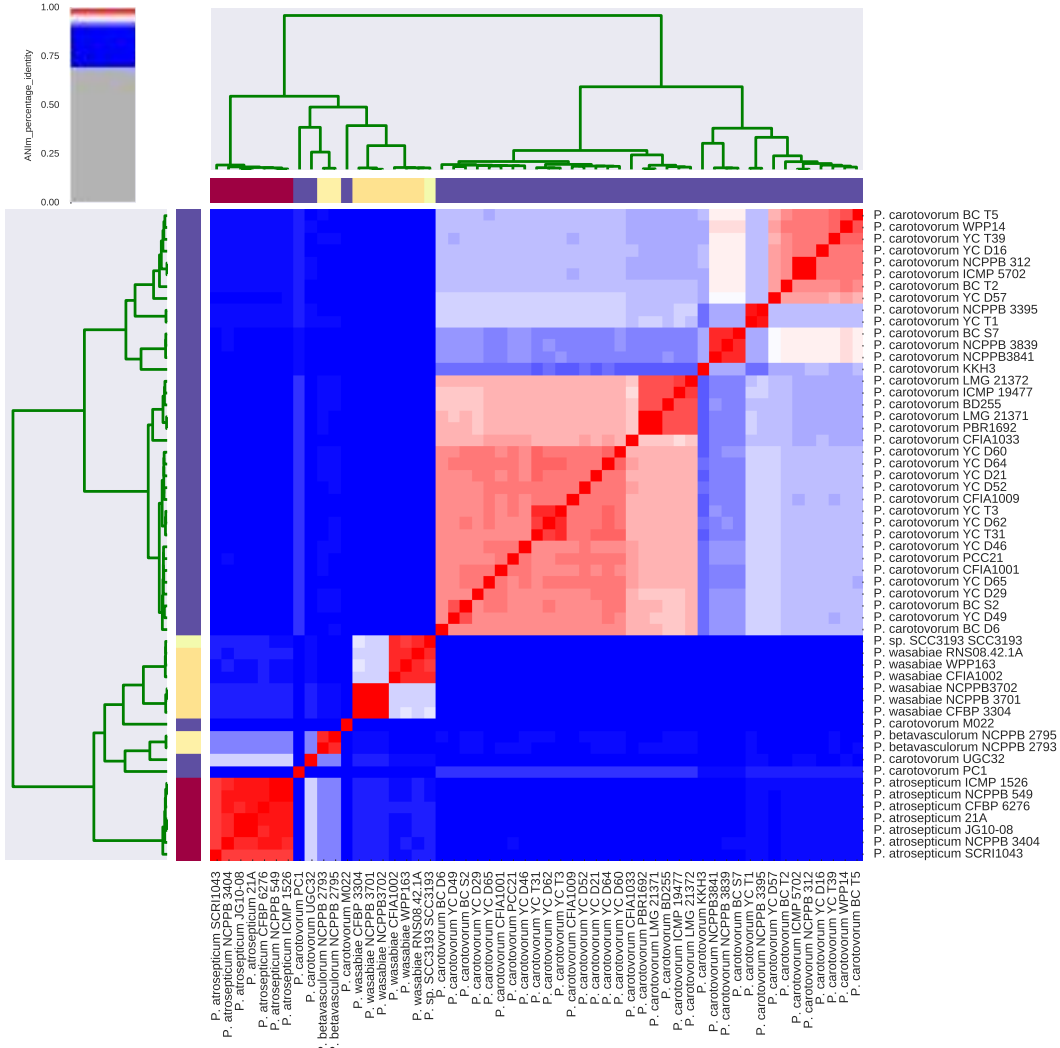
- Ten species-level groups (four novel)
- *P. carotovorum* split: several species - no clean *Pcb*/*Pcc* split
- *P. wasabiae* split: two species



Interpreting ANIm [a]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h

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Criticisms of ANIm

- 95% threshold 'arbitrary'
- Similarity classification, not phylogenetic reconstruction
- No functional (or gene-based) interpretation of risk (cf. pangenome classification and analysis)

ANIm only considers 'homologous' regions

- define 'homologous'
- be misled by HGT/LGT - low total extent of homology?
- is homology phenotypically significant for risk assessment?

Coverage plots help interpretation: exclude HGT/LGT bias.

55 Pectobacterium spp. ANIm[a]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h

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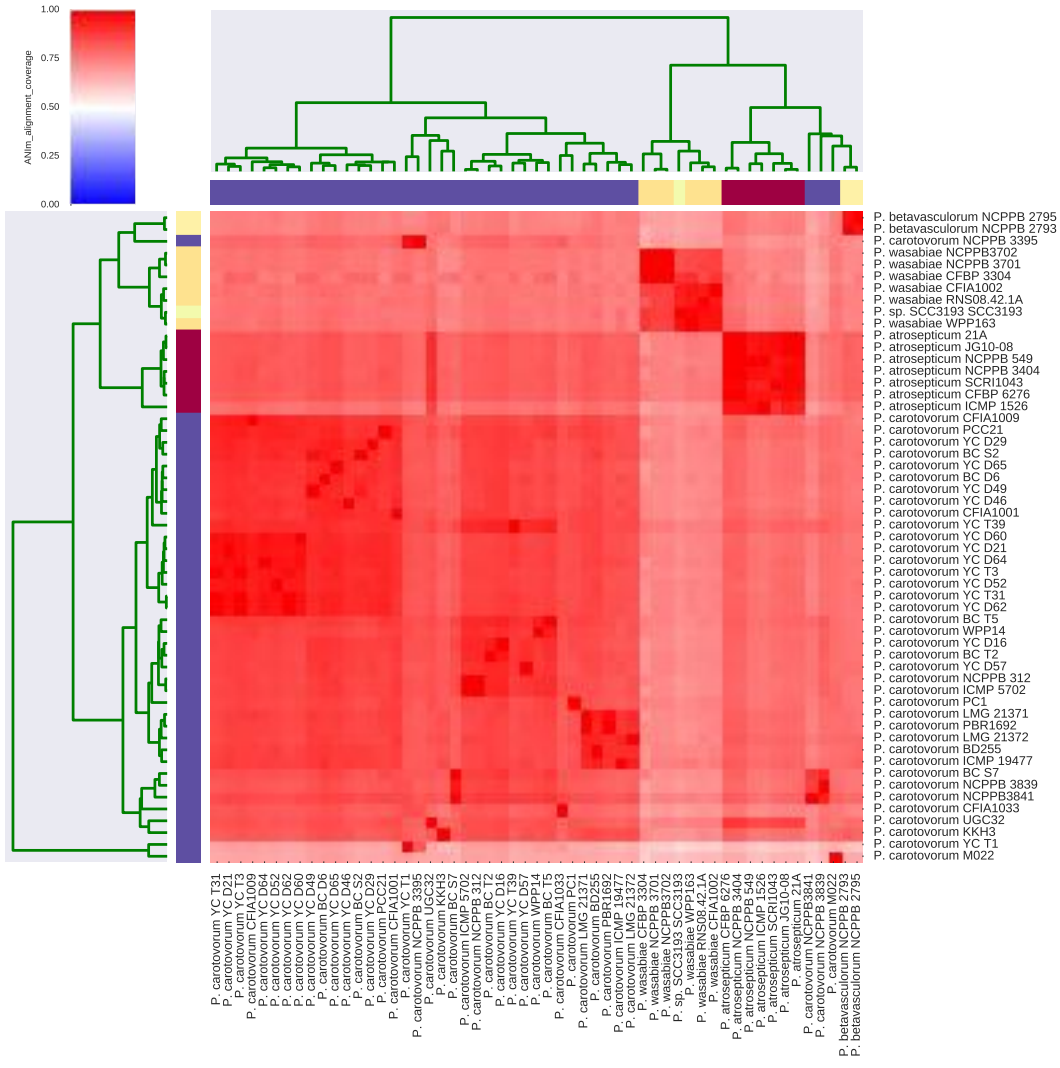
- All isolates align over >50% of whole genome



34 Dickeya spp. ANIm[a]

[a] Pritchard *et al.* (2016) *Anal. Methods* doi:10.1039/c5ay02550h

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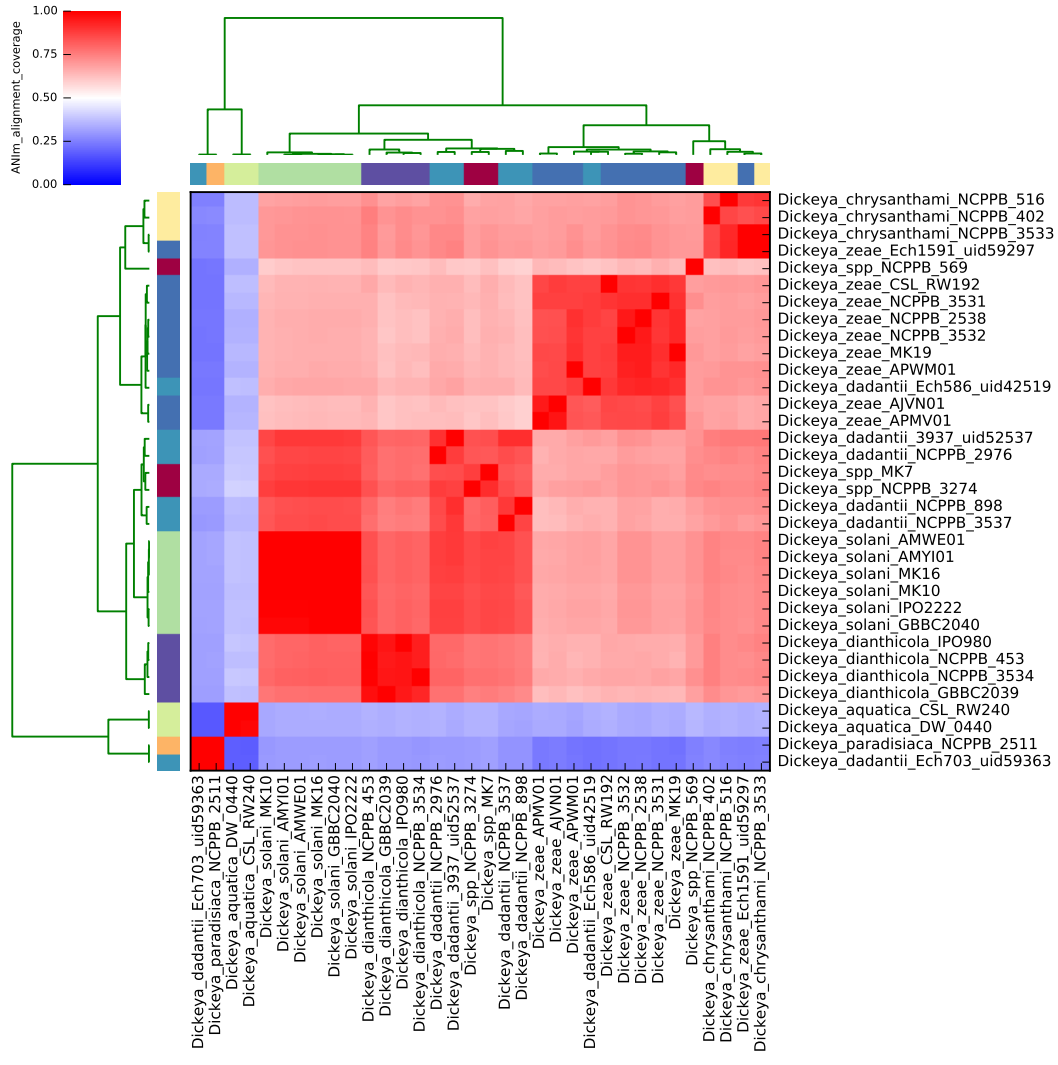
- Most isolates align over >50% of whole genome
- Community: two outlier species are questionable assignments as *Dickeya*



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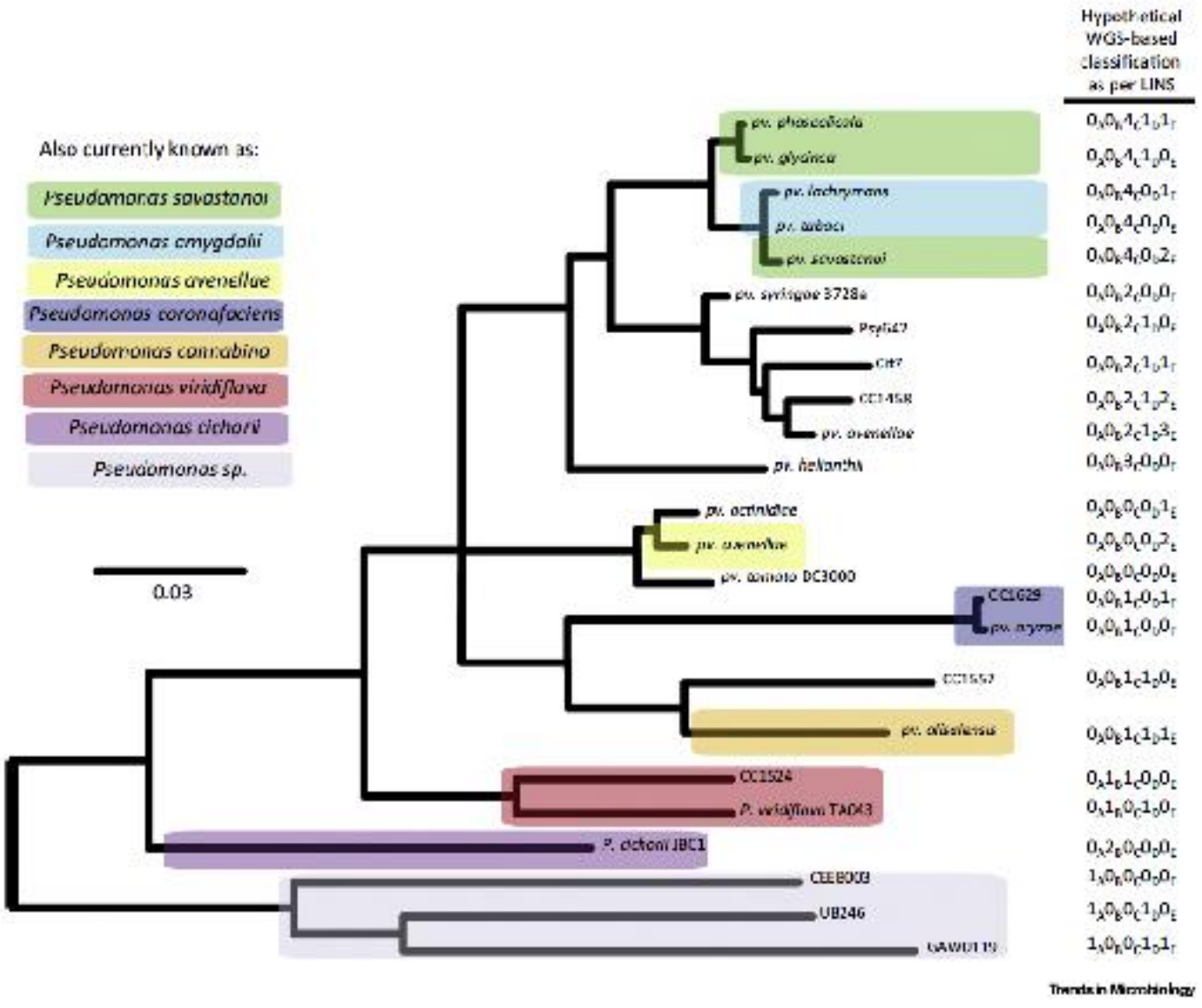
A new classification scheme [a]

[a] Baltrus (2016) *Trends Microbiol.* doi:10.1016/j.tim.2016.02.004



ANIm graphs

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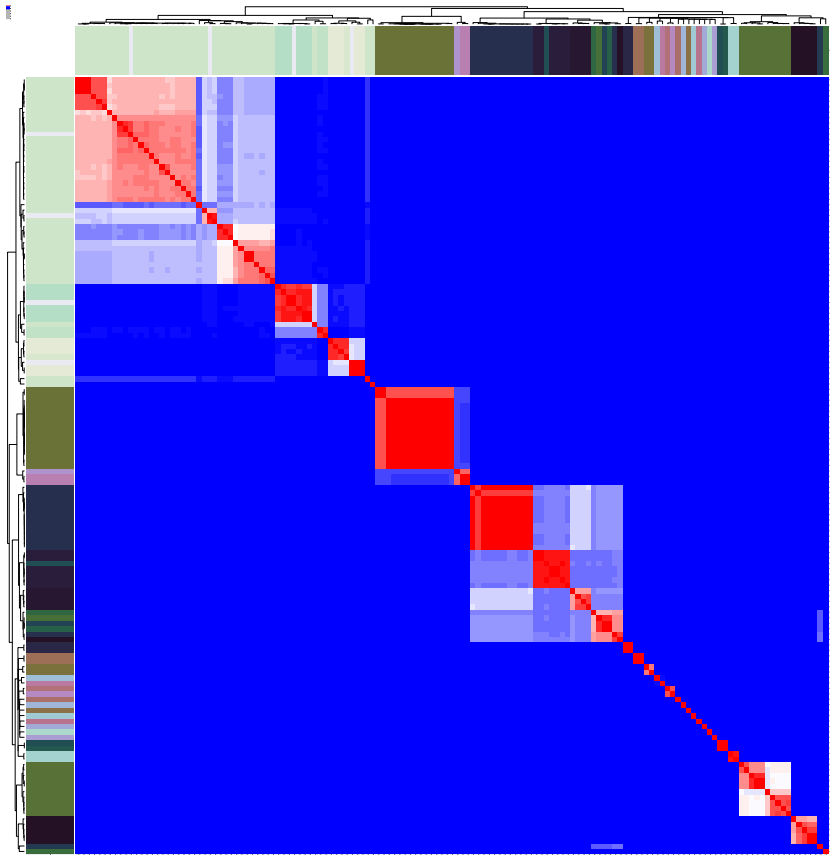
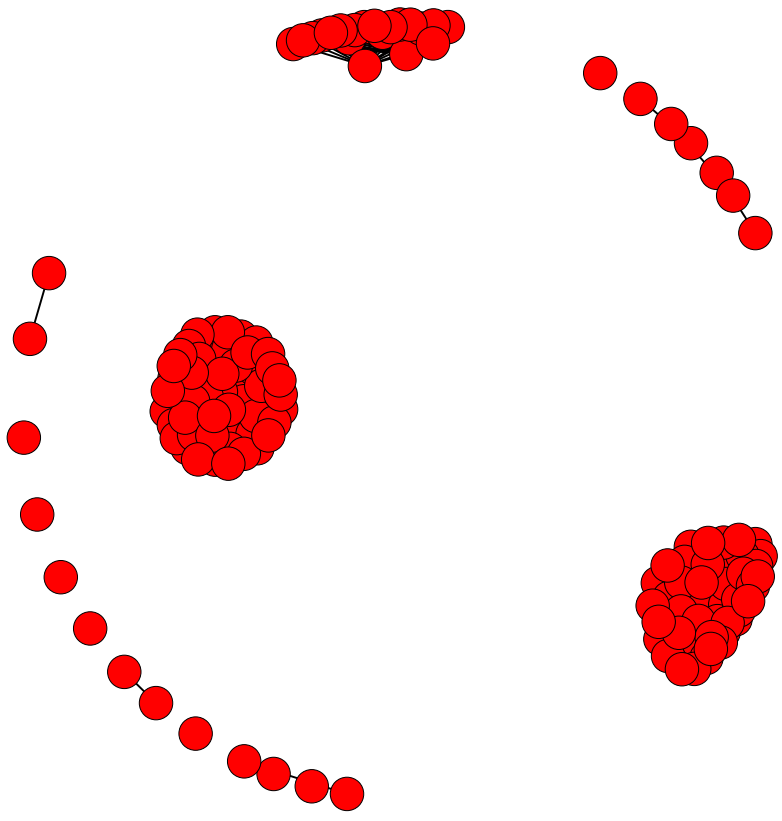
ANIm identity/coverage scores define networks (143 genomes)



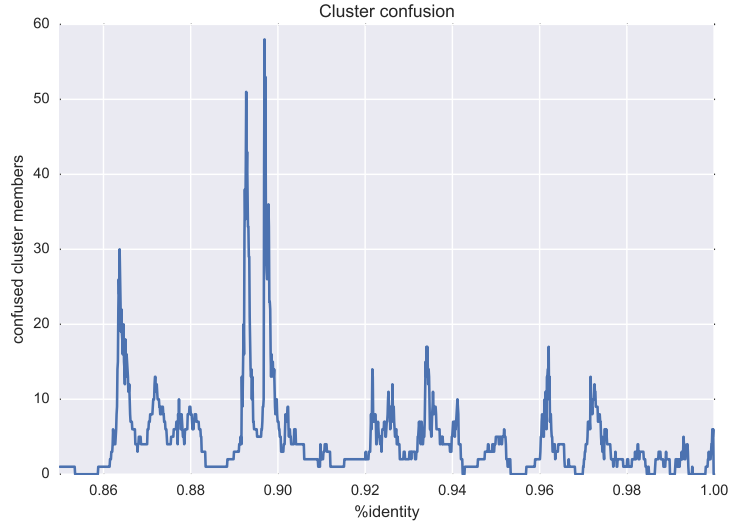


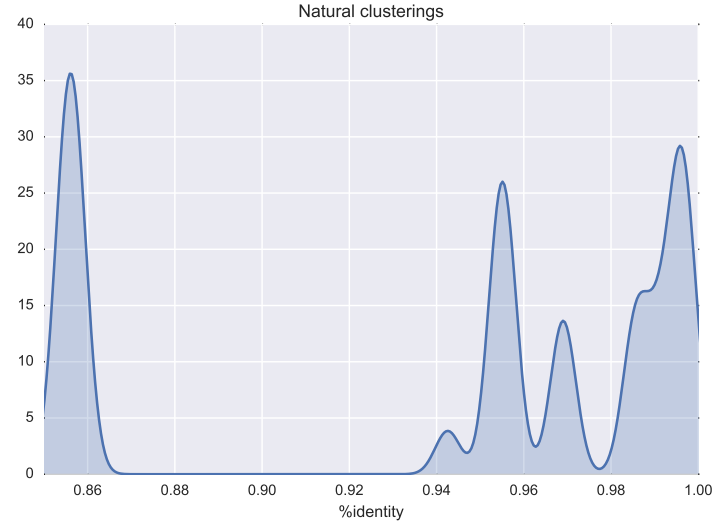
Natural clusterings

Natural clusterings occur in the data - ‘cliques’

‘Clique’ membership varies with %identity

‘Clique’ membership at given %identity a permanent classification





‘Genus’, ‘species’, ‘subspecies’ and ‘clonal’ genomotypes indicated

Genus genomotype

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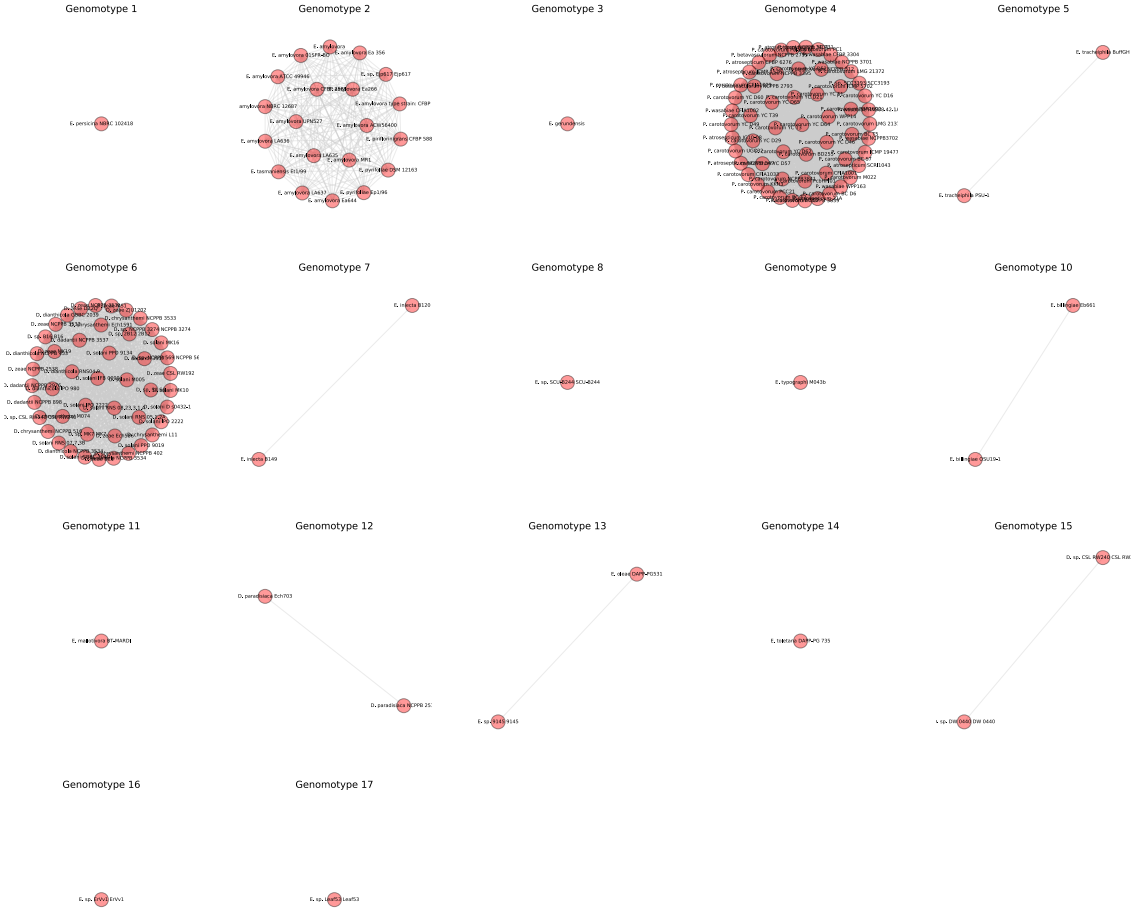
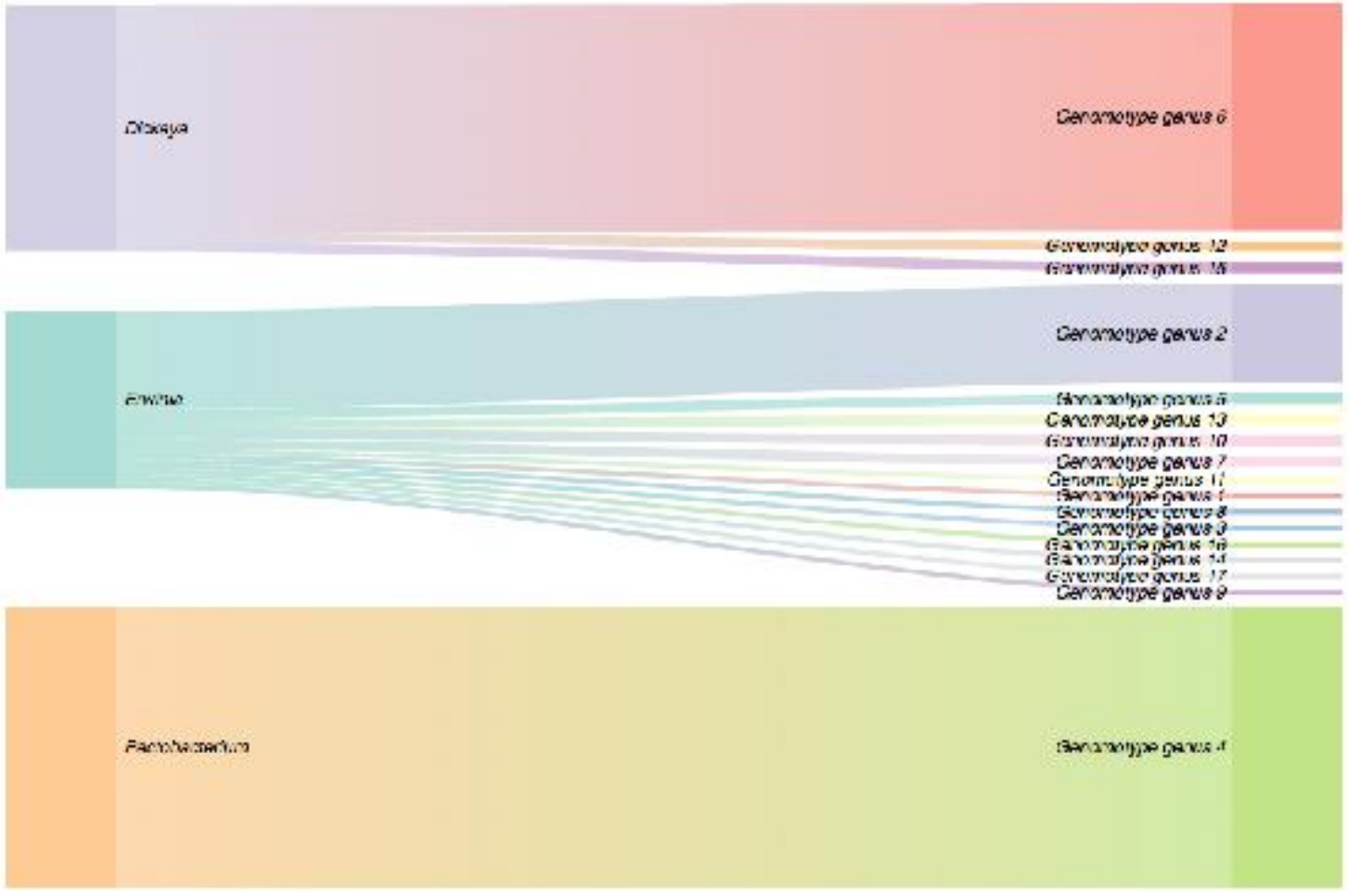
17 genus-level genomotypes indicated



Genus reclassification

Erwinia splits into 13 genomotypes

Dickeya splits into 3 genomotypes



Species genomotype

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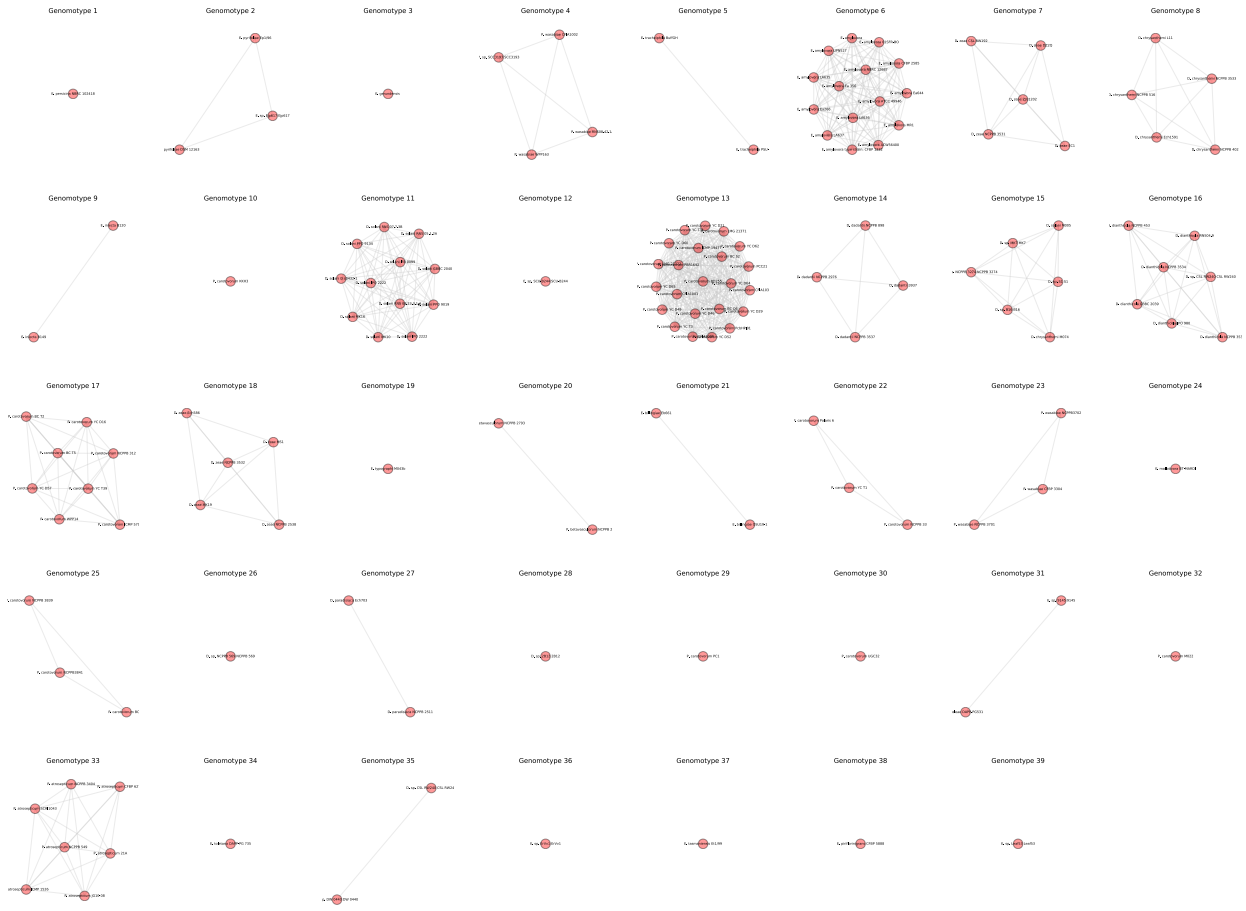
39 species-level genomotypes indicated



Species genomotype

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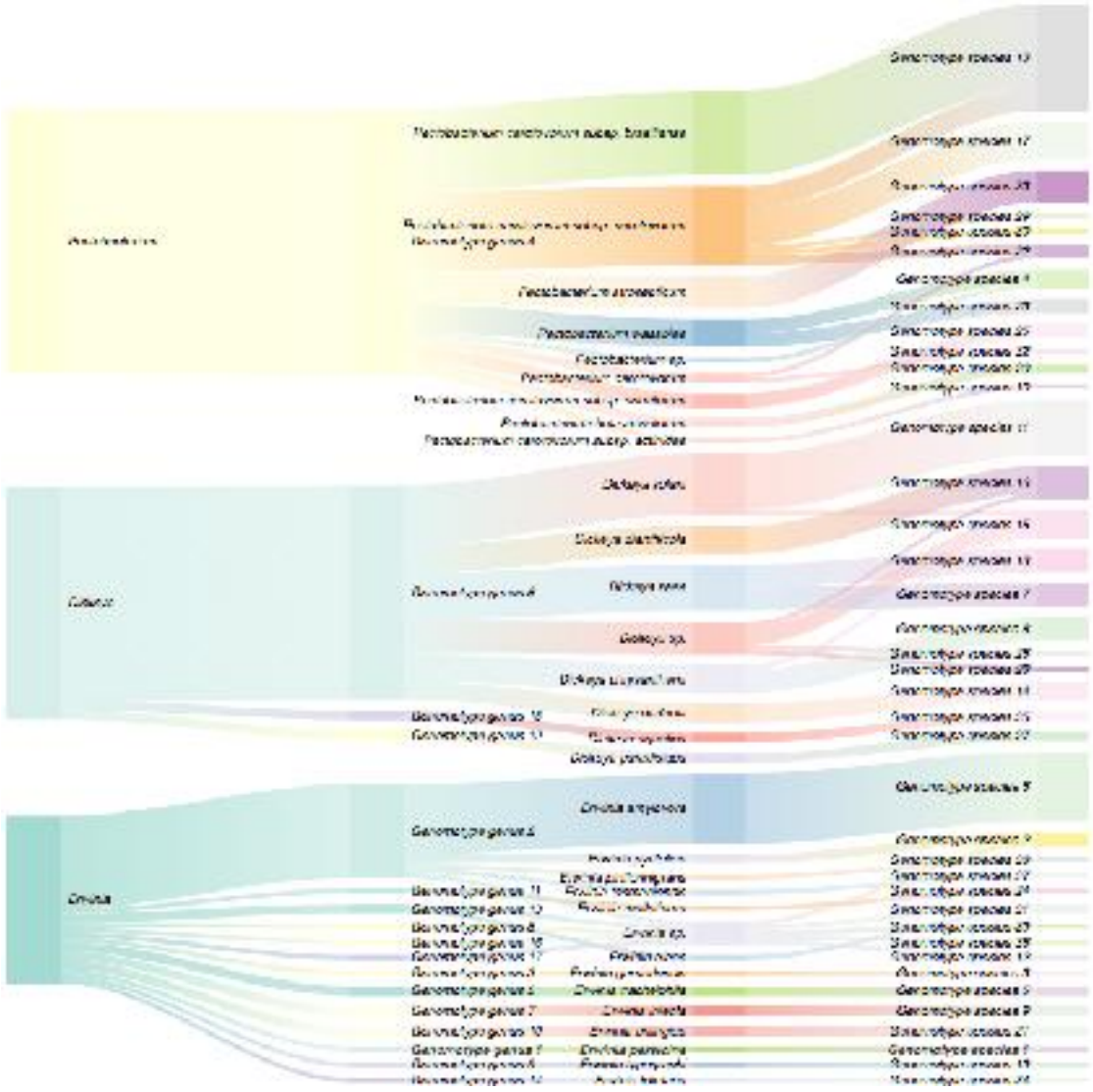
Most species classifications remain unaffected

P. carotovorum subspecies confusion/splits

Suggested reclassifications



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Final thoughts

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Diagnostics and large-scale analyses need accurate classification

- Historical collections/public databases have inaccuracies
- Bacterial taxonomy can be messy!

Whole-genome classification with ANI works

- Retrospective sequencing and classification: clean things up
- Misclassification and hidden diversity may be difficult news for metagenomics...
- Accurate MinION classification in-the-field with ANIm is possible?

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