Validation of real-time PCR assays for the specific detection of each *Xylella fastidiosa* sub-species

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fera

Original thinking... applied

Fera Science Ltd.

‘Our focus is translating research into evidence and science-based products and services’

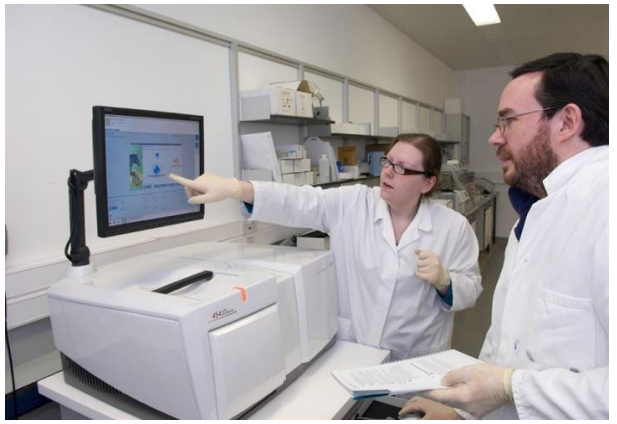
Institute for Plant Pathology, 1914

- 350+ interdisciplinary scientists
- Joint venture between Capita & Defra (UK government)
- Scientific heritage of over 100 years

Department for Environment, Food & Rural Affairs

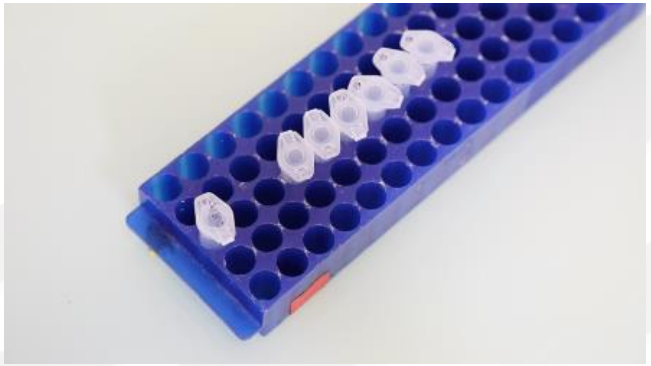
CAPITA

Plant Protection



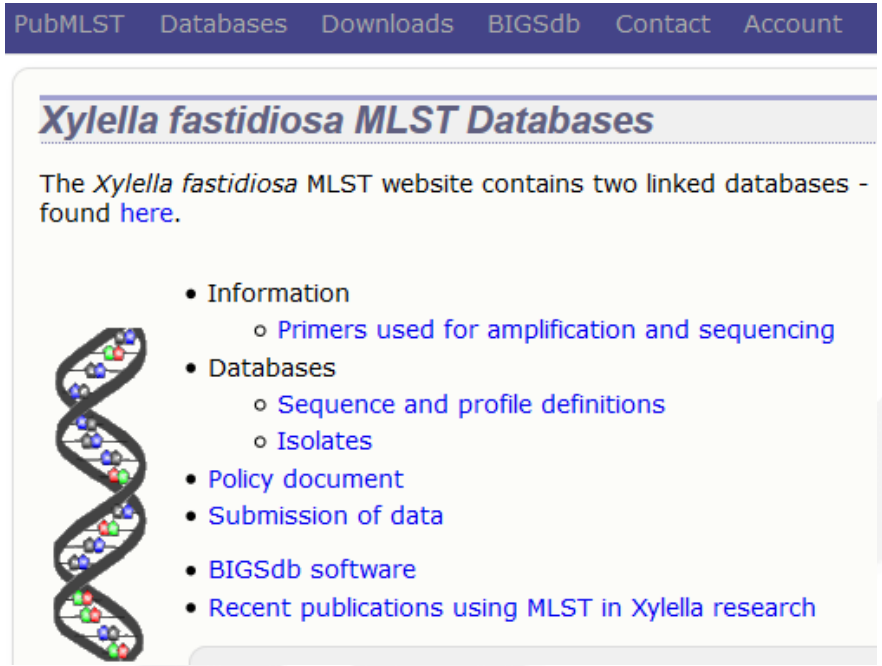
Detection of *Xylella fastidiosa*

- Imports/exports and surveillance
- 500 plant samples a year screened in the lab for the presence of *Xylella fastidiosa*
- Large potential host range
- Isolation is difficult and Xylella is slow growing (10-20 days)
- DNA extracted and tested using real-time PCR



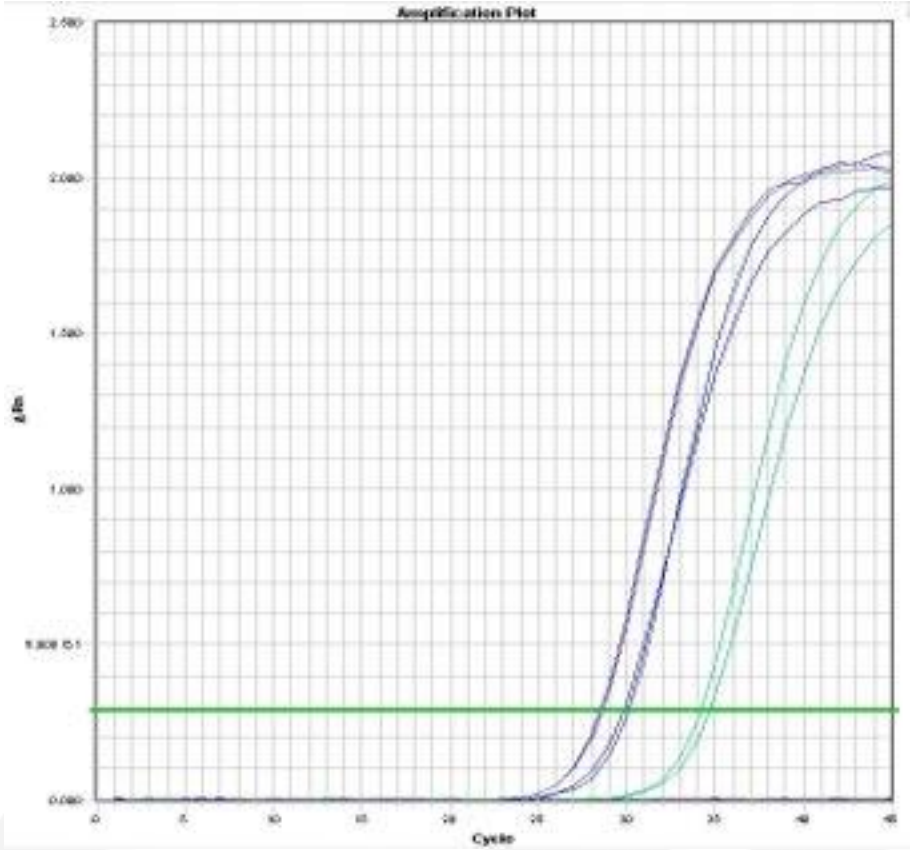
X. fastidiosa sub-species identification

- Many existing assays provide identification to species but not sub-species level (e.g Minsavage *et al.*, 1994)
- Sub-species identification following a positive at species level
- Current method of sub-species identification is MLST, which is time consuming (3+ days)
- Rapid method needed, real-time PCR preferable

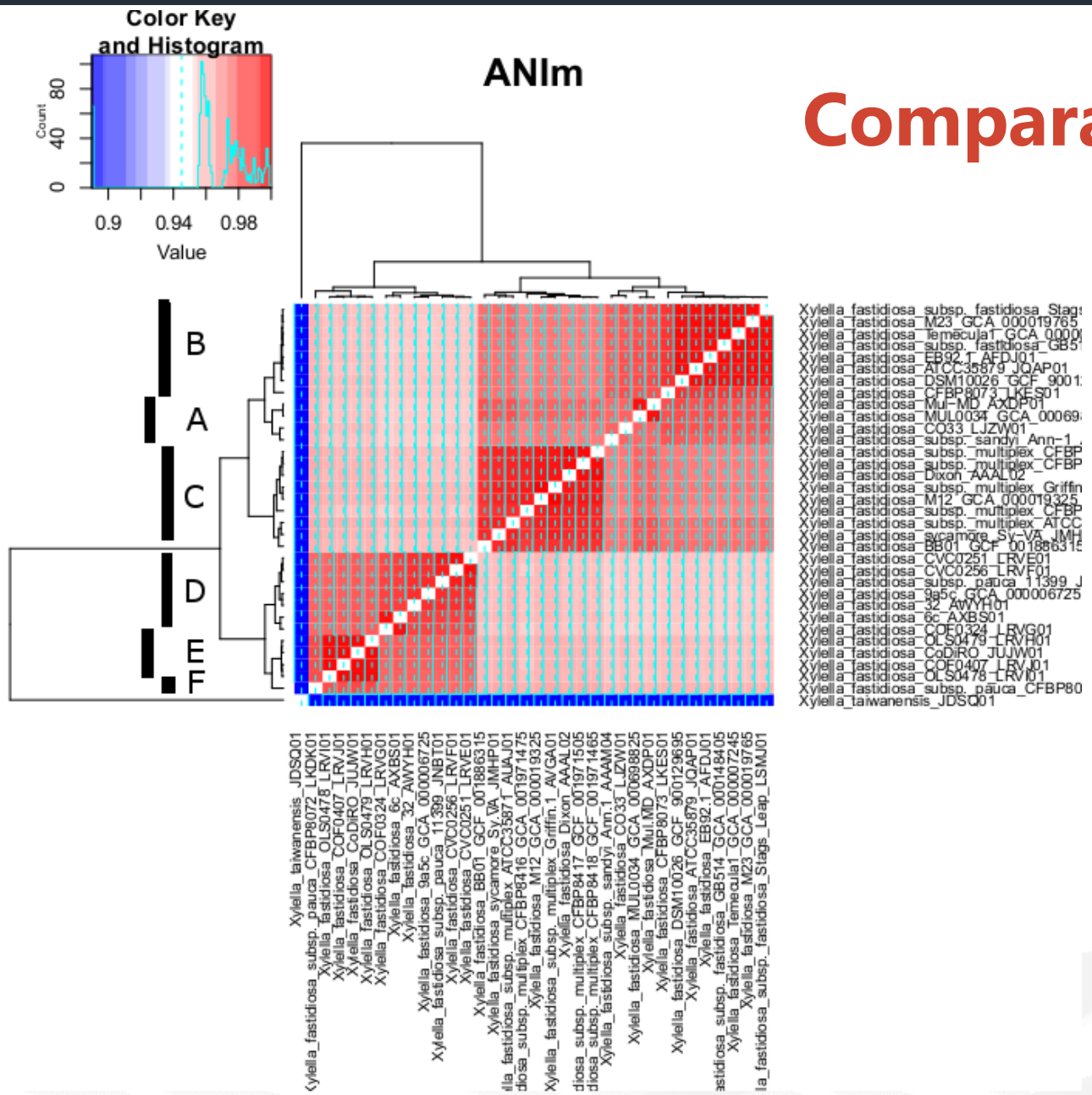


Assay Design methods

- Used a comparative genomics approach on 33 publicly available genomes downloaded from GenBank® (2017) to identify diagnostic markers
- Followed by targeted assay design to develop highly specific diagnostic real-time PCR assays



Comparative genomics



Genome assemblies clustered into 3 major groups, with further sub-divisions.

These groupings are primarily analogous to the formal taxonomic descriptions of *Xylella* subspecies.

X. fastidiosa. subsp. *pauca* is split into 3 clusters and *X. fastidiosa*. subsp. *multiplex* into 2 clusters.

Relevant sub-species	Corresponding sequence cluster
X. f. subsp. *morus*	A
X. f. subsp. *sandyi*	
X. f. subsp. *fastidiosa*	B
X. f. subsp. *multiplex*	C
X. f. subsp. *pauca*	DEF

Heatmap made using gplots package within the statistical software R (www.r-project.org).

Comparative genomic analysis

- Prototype software tool developed to discover sequences unique to each *X. fastidiosa* sub-species = unique markers identified

Sub-species	Unique markers identified	Initial assays designed
X. fastidiosa subsp. *fastidiosa*	125	5
X. fastidiosa subsp. *multiplex*	491	3
X. fastidiosa subsp. *pauca*	6	1
X. fastidiosa subsp. *morus*	45	9
X. fastidiosa subsp. *sandyi*	9	2

Preliminary real-time PCR assay evaluation

- Initial specificity test of all assays demonstrated some were not specific to the desired subspecies- 10 of the 24 assays showed cross-reaction with non-target DNA
- Specificity varied considerably in assays designed to multiple markers

Species / *Xylella* sequence cluster		A-1-Ra	A-1-Rb	B-1-Ra	B-1-Rb	B-2	B-3-Fa	B-3-Fb	C-1	C-2	C-3	DEF-1
X. f. subsp. *morus*	A	15.7	15.4	-	-	-	-	-	-	-	-	-
X. f. subsp. *fastidiosa*	B	-	-	17.5	17.9	16.3	16.7	18.0	-	-	-	-
X. f. subsp. *fastidiosa*	B	-	-	15.8	15.8	12.9	16.1	16.2	-	-	-	-
X. f. subsp. *fastidiosa*	B	-	-	17.9	16.8	16.4	16.0	17.1	-	-	-	-
X. f. subsp. *multiplex*	C	-	-	36.2	34.7	26.0	-	-	27.0	25.1	25.8	-
X. f. subsp. *multiplex*	C	-	-	36.7	-	33.3	-	-	18.2	17.3	18.1	-
X. f. subsp. *pauca*	DEF	-	-	-	-	-	-	-	-	31.6	-	16.9
A. tumefaciens		-	-	-	-	-	-	-	37.4	-	-	-
P. syringae pv. *persicae*		-	-	-	-	-	-	-	-	-	-	-
X. fuscans subsp. *aurantifolii*		-	-	-	-	-	-	-	-	-	-	-
X. campestris pv. *campestris*		-	-	-	-	-	35.1	-	-	-	-	-
NTC (water)		-	-	-	-	-	-	-	-	-	-	-

positive reaction
cross-reaction (non-target)
negative reaction

Assay validation

A clear understanding of performance characteristics of the diagnostic assay is crucial

The assays were validated following EPPO standard PM7/98(2) (EPPO, 2014).

- Analytical specificity and selectivity
- Analytical sensitivity
- Repeatability
- Reproducability

- Assays tested against a large panel of non-target bacterial species including taxonomically closely related species, common plant pathogens, plant/soil endophytes, healthy insects, healthy and infected plant material

Summary of validation data

Analytical sensitivity	Femtograms DNA detected	3 experiments on different days by one user. DNA extracted from pure culture was serially diluted through eight log dilutions in water and tested in 3 technical replicates
Analytical specificity	100% accurate	1-3 targets, 4 other *X. fastidiosa* sub-species, 38 non-target species (50 isolates), 1 insect species (2 specimens), and 13 plant species analysed
Selectivity	No observable impact of host plant	*X. fastidiosa* was detected in 10 different plant genera
Repeatability	High level; 100% Low level; 100% Minimal level; assay dependent (0-62.5%)	8 replicates of DNA from pure culture at varying concentrations; neat DNA (high level), at the assay LOD (low level) and beyond the assay LOD (minimal level) were analysed
Reproducibility	High level; 100%. Low level; 92-100%. Minimal level; assay dependent (8-63%)	Analyses were performed by 2 different operators on 4 different devices on 3 different days with freshly prepared reaction mix. 8 replicates of DNA from pure culture at varying concentrations; neat DNA (high level), at the assay LOD (low level) and beyond the assay LOD (minimal level) were analysed.
Diagnostic sensitivity	100%	11 infected plant samples analysed
Diagnostic specificity	100%	13 non-infected plant samples analysed

Analytical sensitivity

Assay	Limit of detection	C_T with neat DNA	C_T at LOD	R^2 value	Slope	Amplification efficiency
X. f. subsp. *fastidiosa*	124 fg	16.3	35.1	0.9997	-3.4854	0.94
X. f. subsp. *multiplex*	182 fg	16.3	34.4	1	-3.5346	0.92
X. f. subsp. *pauca*	84.2 fg	15.9	34.3	0.9999	-3.5022	0.93
X. f. subsp. *morus*	59.2 fg	17.5	34.8	0.9985	-3.3522	0.99
X. f. subsp. *sandyi*	908 fg	15.6	32.2	0.9998	-3.4142	0.96
Universal (Harper)	124 fg	14.4	31.4	0.9999	-3.3786	0.98

Average values from 3 independent dilution series of DNA (in water), each tested with three technical replicates.
Limit of detection (LOD) defined as the lowest quantity of DNA where 100% of replicates were positive (n=9).

Acknowledgements

- Future Proofing Plant Health Project (Defra)
- Charlotte Howard, Andrew Aspin and John Elphinstone (Fera Science Ltd.)
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BRIGIT

Vector Borne Disease of Plants

Consortium for enhancing UK surveillance and response to *Xylella*

https://www.jic.ac.uk/brigit/

Objectives of BRIGIT

1. Develop the knowledge required:
    a) to reduce risk of *Xylella* being introduced;
    b) to respond to interceptions/outbreaks; and
    c) to mitigate impact if disease becomes established.
2. Deliver good quality science and provide information required by industry, policymakers, academics and citizens in the UK and internationally
3. Develop capacity to respond to other plant pests & diseases

Citizen science & knowledge exchange

- Establish integrated project websites to engage citizens, stakeholders and policymakers – raising public awareness
- Provide accessible information on *Xylella* vectors, including:
  - taxonomy;
  - geographical distribution;
  - plant host range; and
  - sequence

Lead: Ana Perez-Sierra,
Forest Research

Diagnostic capability

Sampling

- Develop understanding of distribution, and rates of colonisation and symptom expression to optimise sampling strategies
- Evaluate novel approaches to target sampling

Detection & Identification

- Harmonise current testing procedures
- Optimise sensitivity of detection in tree hosts
- Evaluate emerging technologies for reliability, sensitivity and specificity of subspecies detection
- Develop tests to identify source of *Xylella* in an outbreak

Lead: John Elphinstone, Fera

Insect vector biology

- Determine location of potential vectors in the environment
- Determine potential for short-range dispersal of meadow froghopper (*Philaenus spumarius*): mark-release-recapture
- Generate genome sequences of potential vectors (froghopper/leafhopper) to assess migration routes and (potential) dispersal between habitats and throughout UK
- Investigation mechanisms underlying insect preferences for specific plant species

Lead: Saskia Hogenhout, John Innes Centre

Epidemiology & modelling

- Assess factors affecting human pathways which move *Xylella*
- Understand purchasing behaviours of consumers, and co-design of identification and detection tools
- Model potential entry and spread within and from the horticultural trade: regulation
- Develop multiscale model of *Xylella* dispersal: surveillance and control

Lead: Steven White, CEH


John Innes Centre


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