Subject: **EUPHRESCO Non-Competitive Project:**

Rapid identification of quarantine bacterial plant pathogens by sequence comparisons within single housekeeping genes.

Start Date: 1 July 2009
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1. Introduction

Monitoring and surveillance of bacterial plant pathogens is vital in efforts to control disease and maintain freedom from alien and newly emerging diseases. Rapid and reliable identification of species and pathovars provides the primary means of evaluating the disease threat from isolated pathogens. With the development and reduced cost of DNA sequencing technologies, phylogenetic-based identification based on comparative sequence analysis of protein coding genes has been shown to be rapid, reliable, reproducible and cost-effective. Multilocus sequence typing (MLST), multilocus sequence analysis (MLSA) and DNA barcoding methods are all now commonly used procedures for identification of bacteria to genus, species and subspecific levels. These methods enable discrimination of species, strains and pathovars that could not previously be reproducibly resolved using traditional phenotype profiling methods, representing a significant advance in pathogen identification.

Initial phylogenetic analyses of plant pathogenic bacteria included identification of the *Ralstonia solanacearu*m species complex (Poussier *et al*., 2000; Fegan and Prior, 2005) for which there are now internationally recognised phylotypes and sequevars identified from 16S-23S rRNA ITS gene sequences, 16S–23S rRNA intergenic spacer (ITS) region sequences and partial endoglucanase (egl) gene sequences (Safni *et al*., 2014). Further published analyses encompass other major plant pathogen groups including *Xanthomonas* species and pathovars (Parkinson *et al*., 2008; Young *et al*., 2008; Bui *et al*. 2009a and 2009b) and *Dickeya* species (Parkinson *et al*., 2009). These studies have indicated that identification based on carefully selected single gene sequences can provide rapid, robust, reliable and cost-effective means for routine identification of bacterial plant pathogens.

Full taxonomic identification using MLST, MLSA or multiple barcoding markers from a series of housekeeping genes can be time consuming and expensive and is not always required for routine identification purposes in the diagnostic laboratory. This work was therefore undertaken to validate a number of protocols, each based on partial sequence analysis of a single gene previously used in more comprehensive phylogenetic analyses, for the routine identification of specific plant pathogenic bacteria of quarantine importance.

2. Project aims and objectives

The aim of the project was to collate, evaluate and format data from published phylogenetic studies and ongoing sequencing studies in European diagnostic laboratories to provide standardised protocols for routine use in diagnostic laboratories for rapid identification of bacterial plant pathogens of statutory importance (including certain *Xanthomonas* spp., pathovars of the *Pseudomonas syringae* complex, *Erwinia amylovora* and *Dickeya* spp).

Specific objectives:

a. Validate published methods for phylogenetic identification of bacterial plant pathogens of plant health importance, including certain *Xanthomonas* and *Dickeya* spp.
b. Finish development of new phylogenetic analyses to allow identification of certain pathovars of *Pseudomonas syringae*. and isolates of *Erwinia amylovora*.
c. Standardise methods for rapid identification of key plant pathogenic bacteria appropriate for use with EPPO diagnostic protocols.
d. Facilitate application of phylogenetic identification schemes in routine diagnostics in collaboration with the EU Qbol project.

3. Project participants

A total of 16 laboratories in 13 countries signed up to the project through their local EUPHRESCO representatives. The participant list is shown in Annex 1.

4. Methods

4.1. Detailed procedures for DNA extraction, amplification and purification of PCR product from bacterial isolates are attached as Annex 2 and were shared amongst all partners. These included published methods for identification of *Xanthomonas* spp. (Parkinson *et al*., 2007 and 2009a) and *Dickeya* spp. (Parkinson *et al*., 2009b). In addition, ongoing construction and publication of the phylogenetic analysis of all pathotypes assigned to the *Pseudomonas syringae* complex, based on partial *RpoD* gene sequences, was completed at Fera (Parkinson *et al*., 2010). Suitable gene targets were also identified at INRA for identification of *Erwinia amylovora* amongst related enterobacteria. The gene targets eventally used for each pathogen group were as follows:

Target organisms	Single gene to be sequenced
Dickeya spp.	*recA*
Erwinia amylovora	*recA*
Pseudomonas syringae	*rpoD*
Xanthomonas spp.	*gyrB*

Fera and INRA provided technical back-up to other laboratories where needed. Liaison with partners in the EC – Seventh Framework QBOL project on DNA barcoding ensured that objectives were compatible and resulting information was shared.

4.2. Partners either generated relevant sequence data from isolates available in their own laboratories or provided DNA and/or isolates of bacteria for amplification of target sequences in other participating laboratories. Sequencing was performed commercially e.g. by Eurofins Genomics (https://ecom.mwgdna.com) or Genoscreen, France (www.genoscreen.fr). Where possible, and depending upon availability of isolates within the consortium, at least 10 isolates per taxa were sequenced. Sequencing was performed on both forward and reverse DNA strands. Pathogens of statutory importance targeted in this study are listed in Table 2.

Table 2: Bacterial pathogens of statutory importance to be identified.

EPPO Listed organism	EPPO diagnostic protocol available
1. *Dickeya dianthicola*	√
2. *Dickeya solani*	
3. *Erwinia amylovora*	√
4. *Pseudomonas syringae* pv. *persicae*	√
5. *Xanthomonas arboricola* pv. *corylina*	√
6. *Xanthomonas arboricola* pv. *pruni*	√
7. *Xanthomonas axonopodis* pv. *allii*	
8. *Xanthomonas axonopodis* pv. *citri*	√
9. *Xanthomonas axonopodis* pv. *dieffenbachiae*	√
10. Xanthomonas axonopodis pv. phaseoli	
11. *Xanthomonas axonopodis* pv. *poinsettiicola*	
12. *Xanthomonas axonopodis* pv. *vesicatoria*	√
13. *Xanthomonas vesicatoria*	√
14. *Xanthomonas fragariae*	√
15. *Xanthomonas translucens* pv. *translucens*	
16. *Xanthomonas oryzae*	√
17. *Xanthomonas oryzae pv. oryzicola*	√

4.3. The resulting DNA sequences were shared and standardised phylogenetic analysis was performed using reference libraries of published sequences in existing sequence databases and incorporating validated sequence from related type and pathotype strains. Sequences were aligned and phylogenetic analysis conducted using MEGA 4: Molecular Evolutionary Genetics Analysis software. The reference libraries were augmented with new sequence data produced from European and worldwide isolates provided by the participating laboratories.

4.4. The final sequence reference library is attached as Annex 3 and has been made available to all partners and to the Qbol project (now represented by the Q-Bank database at http://www.q-bank.eu) for further reference.

5. Results

5.1. *Dickeya dianthicola and Dickeya solani*

Comparison of partial *recA* gene sequence allowed clear differentiation of all isolates of both *Dickeya dianthicola* and *Dickeya solani* from all other species in the *Dickeya* genus (Fig. 1), including *D. dadantii* (subsp. *dadantii* and subsp. *dieffenbachiae)*, *D. chrysanthemi* (pv. *chrysanthemi* and pv. *parthenii*), *D. paradisiaca* and *D. zeae* (phylotypes I and II). All isolates within each taxon clustered together with the single exception of the type strain of *D. dadantii* for which the recA sequence was distinct from all other *D. dadantii* strains studied. A number of strains belonging to potentially new species could also be identified, including those previously reported by Parkinson *et al.* (2009) as *Dickeya* unidentified clades (DUC-2 and DUC-3) and a speciels-level clade (SLC-1) from sugarcane.

European *Dickeya dianthicola* isolates appear almost clonal in lineage, dating back to the 1950's when they were found on *Dianthus* and *Chrysanthemum*, and then on other hosts including *Dahlia*, *Begonia* and eventually chicory and potato. Although mostly originally identified as biovar 1 or 7 *of Erwinia chrysanthemi*, strains of biovar 9 have also been found on *Kalanchoe* but were not differentiated from other strains of *D. dianthicola* by recA sequence analysis. Similarly, isolates of *D. solani* collected from potato in different locations formed a single sequevar, also indicating a clonal lineage for this pathogen.

5.2. *Erwinia amylovora*

All reference strains and other isolates of *E. amylovora* studied had identical recA sequence and are assumed to have evolved through a single phylogenetic lineage (Fig. 2). They were clearly differentiated from reference strains of their closest (non plant pathogenic) relatives by this method.

5.3. *Pseudomonas syringae* pv. *persicae* and *P. syringae* pv. *actinidiae*

Reference strains, including type strains of all species and pathovars within the *Pseudomonas syringae* complex, clustered into 7 distinct phylogroups according to *rpoD* sequence similarity (Fig. 3). These phylogroups coincide with previously published genomospecies (Gardan *et al*., 1999). Full details of relatedness within the *P. syringae* complex according to partial rpoD sequence have been published elsewhere (Parkinson *et al*., 2011).

According to *rpoD* sequence similarity, the pathotype strain of EPPO A-2 listed *Pseudomonas syringae* pv. *persicae* clustered with other strains in phylogroup 1 (eqivalent to genomospecies 3). This phylogroup comprises a numer of other pathotypes of *P. syringae*, including *P. syringae* pv. *morsprunorum* and *P. syringae* pv. *avii*, both of which cause disease on *Prunus* but are not listed quarantine pathogens. Analysis of *rpoD* sequences from a wider range of isolates from nut and stone fruit hosts (Parkinson *et al*., 2010) indicates that it is possible to differentiate clonally related strains of *P. syringae* pv. *persicae* from *P. syringae* pv. *morsprunorum* (Fig. 4). However, *P. syringae* pv. *persicae* appears to have evolved from the same lineage as *P. syringae* pv. *avii* and the two pathogens are indistinguishable on the basis of *rpoD* sequence alone. Furthermore, it is not possible to distinguish *P. syringae* pv. *morsprunorum* from *P.*

avellanae, a pathogen of hazel (*Coylus avellana*). Since *P. avellanae* is the only species type strain in phylogroup1, further taxonomic investigation is required to determine whether members of this phylogroup, including *P. syringae* pv. *persicae*, should be renamed as *P. avellanae*.

The EPPO A-2 listed pathogen *P. syringae* pv. *actinidiae* may also be identified on the basis of unique *rpoD* sequence as an apparent clonal lineage within phylogroup 1, from which the tea pathogen *P. syrinage* pv. *theae* also appears to have evolved and shares identical rpoD sequence. The other alert-listed pseudomonad *P. syringae* pv. *aesculi* clusters with a large number of closely related pathogens in phylogroup 3, equivalent to genomospecies 2 of Gardan *et al.* (1999).

A number of isolates from diseased prunus (AGES544-01 and AGES145-00 from Austria and ISPaVe1447 from Italy) were sucessfully characterised as part of this study as members of phylogroup 2 (genomospecies 1) together with *P. syringae* pv. *syringae*. Other isolates from Prunus (AGES274-88, AGES283-88 and AGES550-90) were confirmed not to be members of the *P. syringae* complex.

5.4. *Xanthomonas arboricola* pvs. *corylina* and *pruni*

On the basis of partial *gyrB* sequence similarity, all reference strains and isolates of *Xanthomonas arboricola* clustered within a single clade (Fig. 5). Furthermore, all 32 *Xanthomonas arboricola* pv. *pruni* isolates studied formed a single sequevar. Isolates submitted from Italy as *X. arboricola* pathovars *juglandis* (ISPaVel285 and ISPaVel164) and *corylina* (ISPaVel150), as well as isolate LMG 12707 (submitted as *X. oryzae* pv. *oryzae*) also clustered within this sequevar, suggesting a need for further verification of their original identification. A single isolate (NCPPB 1826) obtained from *Prunus domestica* in the UK in 1966 had *gyrB* sequence identical to that of the species type strain of *X. arboricola* pv. *juglandis* (NCPPB 411). Isolates of *X. arboricola* pv. *corylina* (NCPPB 935, ISPa were distinguished from all *X. arboricola* pv. *pruni* isolates studied but not from reference strains of other *X. arboricola* pathovars. A number of unidentified *Xanthomonas* isolates from *Chrysanthemum* (NCPPB 2856 2864, 2865, 2866 and 3200) and a clove isolate (NCPPB 3218) also clustered together with pathovars of *Xanthomonas arboricola*.

5.5. *Xanthomonas axonopodis* pv. *allii*

Seven reference isolates of *X. axonopodis* pv. *allii* all clustered together in a single clade grouping a large number of closely-related *Xanthomonas* pathogens with highly similar *gyrB* sequence, including the type strains of 3 species; namely *X. perforans*, *X. alfalfae* and *X. euvesicatoria* but not the type strain of *X. axonopodis* (Fig. 5). Although distinguished from other members of this clade on the basis of minor *gyrB* sequence differences, four reference isolates of *X. axonopodis* pv. *alli* shared identical sequence with a single isolate identified as *X. alfalfae* subsp. *citrumelonis*.

5.6. *Xanthomonas axonopodis* pv. *citri*

All isolates studied of *Xanthomonas axonopodis* pv. *citri* (synonyms *Xanthomonas citri* subsp. *citri* and *Xanthomonas citri* pv. *citri*) clustered within a single clade on the basis of *gyrB* sequence similarity (Fig 5). Sequence diversity within this clade is insufficient to differentiate isolates of *X. axonopodis* pv. *citri* from a large number of other pathovars of *X. axonopodis*, *X. citri* or *X. fuscans*. Nevertheless, all *xanthomonads* pathogenic to *Citrus* clustered within the single clade with the exception of *Xanthomonas alfalfae* subsp. *citrumelonis. Citrus* isolates of the closely related *X. fuscans* subsp. *aurantifoliae* (synonym *X. axonopodis* pv. *aurantifoliae*) all cluster in a single sub-clade. Some isolates submitted as *X. axonopodis* pv. *citri* which also cluster in this sub-clade (CFBP2866, CFBP2901, Xc148, Xc341) may require further investigation to determine whether they may in fact be *X. fuscans* subsp. *aurantifoliae*.

5.7. *Xanthomonas axonopodis* pv. *dieffenbachiae*

All isolates studied of *X. axonopodis* pv. *dieffenbachiae* clustered together and were easily differentiated from closely related plant pathogenic xanthomonads and isolates of *Xanthomonas citri* pv. *dieffenbachiae* on the basis of *gyrB* sequence similarity (Fig. 5).

5.8. *Xanthomonas axonopodis* pv. *phaseoli*

A number of known reference strains of *X. axonopodis* pv. *phaseoli* clustered with the pathotype strain (NCPPB 3035 = LMG 7455) within a clearly identifiable clonally related group of strains which also contained reference strains of *X. axonopodis* pv. *manihotis* (NCPPB 1834) and *X. campestris* pv. *passiflorae* (NCPPB 2346) as well as an unidentified Japanese *Xanthomonas* isolate from pea (NCPPB 3761). The type strain of *X. axonopodis* was not contained within this lineage (Fig. 5).

Other xanthomonads isolated from *Phaseolus* were identified as a single clone which included the type strain of *X. fuscans* (syn. *X. citri* pv. *phaseoli* var. *fuscans*) but which clustered together with a highly related group of diverse xanthomonad pathovars also containing the type strains of *X. citri* and *X. axonopodis*.

Two other isolates from *Phaseolus* (LMG 829 and LMG 8013) clustered as a distinct sequevar together with the pathotype strain of *X. campestris* pv. *malloti* from *Mallotus japonicus* (NCPPB 4347) and their original identification as *X. axonopodis* pv. *phaseoli* could therefore not be confirmed.

5.9. *Xanthomonas axonopodis* pv. *poinsettiicola*

Sequence comparisons within the *gyrB* locus (Fig. 5) grouped three reference strains isolated from *Euphorbia* in a single lineage. These included the pathotypes of *X. axonopodis* pv. *poinsettiicola* (NCPPB 581) and *X. campestris* pv. *euphorbiae* (NCPPB 1828) and an Austrian *Euphorbia* isolate, none of which could be distinguised on the basis of *gyrB* sequence. The species type strains of *X. alfalfae*, *X. euvesicatoria* and *X. perforans* also grouped within the same lineage as did a large diversity of other pathovars affecting non-*Euphorbia* hosts.

5.10. *Xanthomonas vesicatoria* and *Xanthomonas axonopodis* pv. *vesicatoria*

The species type strain (NCPPB 442 = LMG 911) and other reference strains of *X. vesicatoria* were clearly identified by a clonal lineage of identical *gyrB* sequence (Fig. 5). All other xanthomonads isolated from *Lycopersicon* or *Capsicum* and originally identified as either *X. campestris* pv *vesicatoria*, *X. axonopodis* pv. *vesicatoria*, *X. euvesicatoria* or *X. perforans* were differentiated from *X. vesicatoria* and clustered in the *X.alfalfae/euvesicatoria/perforans* lineage with the exception of a single tomato isolate (*X. campestris* pv. *vesicatoria* LMG 927) which grouped with the *X. campestris* type strain (NCPPB 528). The type strain of *X. gardneri* (NCPPB 881 = LMG 962), isolated from tomato in the former Yugoslavia, clustered with the type strains of *X. cynarae* and *X. hortorum* on the basis of *gyrB* sequence similarity and was therefore clearly differentiated from all of the above tomato pathogens.

5.11. *Xanthomonas fragariae*

All submitted isolates and reference strains of *X. fragariae* shared identical *gyrB* sequence with the type strain (NCPPB 1469) and could be easily differentated from all other plant pathgenic xanthomonads (Fig. 5).

5.12. *Xanthomonas translucens* pv. *translucens*

All reference strains of *X. translucens* clustered together and could be clearly identified from all other plant apthogenic xanthomonads on the basis of partial *gyrB* sequence (Fig. 5). All isolates of the quarantine pathogen *X. translucens* pv. *translucens* shared identical gyrB

sequence with the pathotype strain (NCPPB 973) as well as with isolates of pathovars *hordeii*, *secalis* and *undulosa* but not with the other described pathovars of the species.

5.13. *Xanthomonas oryzae* pvs. *oryzae* and *oryzicola*

All submitted isolates and reference strains of *X. oryzae* clustered together and could be differentiated from all other plant pathogenic xanthomonads on the basis of gyrB sequence similarity (Fig. 5). All submitted and reference strains of *X. oryzae* pv. *oryzicola* clustered together and could be clearly identified with the exception of one (MAI 11) which may warrant further work to verify the original identification. More strain diversity was identified within *X. oryzae* pv. *oryzae*, where a number of *gyrB* sequevars were apparent.

6. Discussion

The increasing speed and reduced cost of nucleotide sequence determination, together with improved web-based databases and analysis tools, present the prospect of increasingly wide application of DNA sequence-based identification methods in routine diagnostic laboratories. Multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) involving sequences from multiple housekeeping genes, or even full genome sequence comparisons, now offer highly accurate taxonomic classification of new isolates of plant pathogenic bacteria and insights into their relatedness and evolution. For more rapid and less expensive routine identifications, DNA barcoding methods allow delineation of specific organisms or taxa based on short genomic sequences previously verified to be specific to the target organism(s) in question. This project aimed to investigate the value of potential barcode sequences within selected single target genes to identify plant pathogenic bacteria of statutory improtance within the EU.

Application of a number of selected methods was demonstrated across a wide diversity of European diagnostic laboratories in 13 EU Member States. Development and harmonisation of standardised procedures increased awareness of their simplicity, reproducibility and cost efficiency. Sequence data generated and analysed during the course of the project was used to evaluate the value of the procedures for rapid identification of a wide diversity of key plant pathogenic bacteria. In many cases, the various target organisms were readily identified on the basis of partial sequence comparisons from only one of three target genes (*recA*, *rpoD* or *gyrB*). However, for some target taxa within the genus *Xanthomonas*, such as those causing disease on citrus, sequence diversity within the *gyrB* gene is insufficient to differentiate isolates of *X. citri* pv. *citri* from a large number of other pathovars of *X. axonopodis*, *X. citri* or *X. fuscans*. In such cases, it will be necessary to identify additional barcoding genes to increase the level of discrimination. Such investigation will be undertaken within the EU Q-bol project to which all sequence data arising from this project has been made available.

7. References

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ANNEX 1: SHORT TOPIC DESCRIPTION AND PARTICIPANTS

Phylogenetic Identification of Quarantine Bacterial Plant Pathogens

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ANNEX 2: STANDARDISED PROTOCOLS

1. *Pseudomonas syringae* complex.

A recent seven locus phylogenetic population study of *P. syringae*-related pathogens (Sarker and Guttman, 2004; Sarkar et al., 2006), identified five major taxa. The primers suggested below amplify a longer *rpoD* locus than used in the previous study and amplify all ten species currently associated with the complex including *P. viridiflava*.

DNA purification.

Purify DNA from freshly grown colonies on nutrient agar dextrose plates. Chelex resin purification has proven to be reliable for all Gram-negative organisms.

Chelex 100 preparation:

Resuspend 1.5 g Chelex 100 (BioRad 142-2832) in 25 ml H_2O (not TE buffer) to make a 6% solution, which can be used directly or stored after autoclaving. A 1ml pipette is used to aliquot the Chelex, which is maintained in suspension using a magnetic stirrer at moderate speed.

1) Prepare 1 ml cell suspensions to between OD_{650}, 0.1-0.2 and micro-centrifuge for 5 min at 10 000 x g.
2) Discard the supernatant carefully by using a pipette.
3) Resuspend the pellet in 300 µl of Chelex suspension (see above) by vortexing and incubate in a water bath at 56°C for 20 min.
4) Vortex at high speed for 10 seconds. Place the microfuge tube in a heated block at 100°C for 8 min. Note: Fix the caps using a weight to avoid the caps opening (explosively!) during heating.
5) Vortex tubes at high speed for 10 seconds, and immediately chill on ice.
6) Centrifuge the tube for 5 min at 14000 x g. Transfer 200 µl of the supernatant carefully to a new micro-centrifuge tube.
7) Use 2 µl aliquot of the supernatant as template DNA in a 50ul PCR reaction.

Primers
PsrpoD FNP1 5'-TGAAGGCGARATCGAAATCGCCAA-3'
PsrpoDnprpcr1 5'-YGCMGWCAGCTTYTGCTGGCA-3

***rpoD* PCR amplification**.

A standard PCR reagent mix is used with final reagent concentrations of 2.5 mM $MgCl_2$, 300nM each primer, 0.2 mM dNTPs and 1 unit Taq polymerase in a final volume of 25 µl. Cycling parameters are: Initial denaturation 94°C/2 min, followed by 34 cycles with annealing at 57°C/30 sec, extension at 72°C/30 sec and denaturation at 94°C/45 sec. Reactions were completed at 72°C/7 min.

Template purification and sequencing

It is recommended to visualise the yield and purity of the amplification product using standard gel electrophoresis methods. The approximately 700 base pair amplicon is purified using commercially available kits (eg. from Promega or Qiagen), to remove excess nucleotides, buffer and enzyme. After reconstituting in water the templates are ready for sequencing. The PCR primers work efficiently as sequencing primers.

MWG/Eurofins provide a commercial sequencing service. Details for sending the purified PCR product and sequencing primers are provided on their web site at https://ecom.mwgdna.com. Pre-paid 'value read' sequencing labels can be obtained in lots of 50, which has the advantage of allowing any number of samples to be sent over any time period without requiring separate invoices. Automation reduces costs and requires the tubes containing the template (7.5 µl of purified PCR product) to be labelled with one of the prepaid labels, which has a unique barcode. Also include separate tubes

containing 4 µl of each forward and reverse primer (2 pmol per µl) for each PCR product to be sequenced. Details of the template and sequencing primer are supplied by logging on to the 'value read page' prior to dispatch of the template and primers. Sequences with the details supplied are then returned by email, usually within 48 hours.

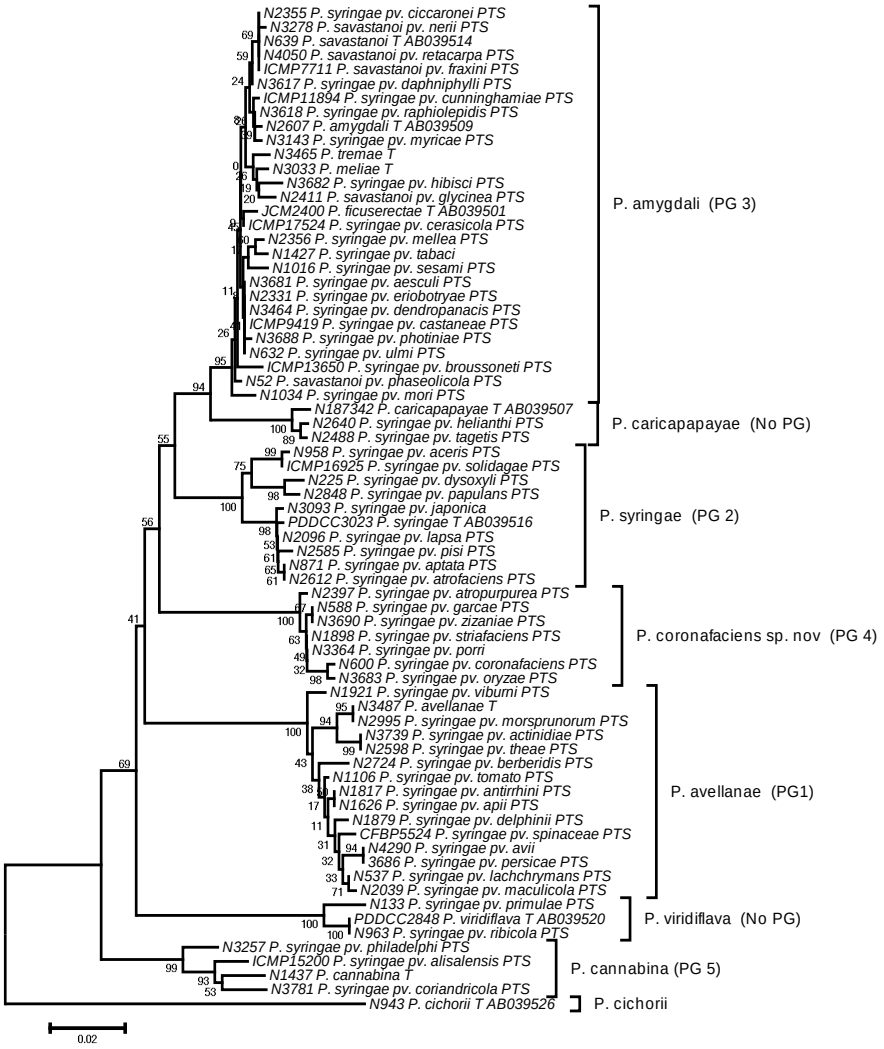
Phylogenetic analysis and reference sequence files

Alignments using Clustal and neighbour-joining phylogenies are produced using standard methods. The freely available MEGA phylogenetics package is most often used (http://www.megasoftware.net). Files containing previously sequenced reference sequences will be supplied to indicate positions for clipping to standard nucleotide start and finish positions.

References

Sarkar, S. & Guttman, D. (2004) Evolution of the core genome of Pseudomonas syringae, a highly clonal, endemic plant pathogen. *Appl Environ Microbiology* **70**, 1999-2012

Sarkar, S., Gordon, J., Martin, G. & Guttman, D. (2006) Comparative genomics of host-specific virulence in *Pseudomonas syringae*. *Genetics* **174** 1041-1056



Phylogeny based on partial *rpoD* sequence comparisons of *Pseudomonas syringae*-related species type strains (STS) and pathovar type strains (PTS). Phylogroups (PG) are as previously described (Sarkar *et al*., 2006).

2. *Dickeya* spp.

The *recA* primers indicated below have been used to produce a population phylogeny including all NCPPB *Dickeya* holdings (Parkinson *et al*., 2009). All recognized taxa were resolved as well as a species-level clade (SLC-1) and two other unclassified clades (DUC-2 and DUC-3). Two primer sets (W=Waleron *et al*., 2002 and P=Parkinson et al., 2009), each with different PCR cycling parameters are available. We recommend to initially use the W set, which produces a longer amplification product, and use the P set if strains are encountered which fail to produce a PCR product.

DNA purification.

We purify DNA from freshly grown colonies on nutrient agar dextrose plates. Chelex resin purification has proven to be reliable for all Gram-negative organisms.

Chelex 100 DNA preparation:

Resuspend 1.5 g Chelex 100 (BioRad 142-2832) in 25 ml H_2O (not TE buffer) to make a 6% solution, which can be used directly or stored after autoclaving. A 1ml pipette is used to aliquot the Chelex, which is maintained in suspension using a magnetic stirrer at moderate speed.

1) Prepare 1 ml cell suspensions to between OD_{650}, 0.1-0.2 and micro-centrifuge for 5 min at 10 000 x g.
2) Discard the supernatant carefully by using a pipette.
3) Resuspend the pellet in 300 µl of Chelex suspension (see above) by vortexing and incubate in a water bath at 56°C for 20 min.
4) Vortex at high speed for 10 seconds. Place the microfuge tube in a heated block at 100°C for 8 min. Note: Fix the caps using a weight to avoid the caps opening (explosively!) during heating.
5) Vortex tubes at high speed for 10 seconds, and immediately chill on ice.
6) Centrifuge the tube for 5 min at 14000 x g. Transfer 200 µl of the supernatant carefully to a new micro-centrifuge tube.
7) Use 2 µl aliquot of the supernatant as template DNA in a 50ul PCR reaction.

PCR Primers

W primer set
RecAF 5'-GGTAAAGGGTCTATCATGCG-3'
RecAR 5'-CCTTCACCATACATAATTTGGA-3'

P primer set

DIC.CONFPCR1	5'-ATCTCCACGGGCTCCCTGTC-3'
DIC.CONRPCR1	5'-ATRCGRCGRCGRATRTCCAGACGGAC-3'
DIC.CONFSP1	5'-CACGGGCTCCCTGTC-3'
DIC.CON.RSP1	5'-CGRATRTCCAGACGGAC-3'

***RecA* PCR amplification**.

A standard PCR reagent mix is used, final reagent concentrations were $MgCl_2$ 2.5 mM, primers 300nM each, dNTPs 0.2 mM and Taq polymerase 1 unit in a final volume of 25 µl. Cycling parameters for the W primer set are: Initial denaturation 95°C /3 min, followed by 34 cycles annealing at 47°C/1 min, extension at 72°C/2 min and denaturation at 94°C/1 min. Reactions were completed at 72°C/5 min. This produces an amplification product of approximately 700 base pairs.

For the P set: Initial denaturation 95°C/3 min, followed by 34 cycles annealing at 55 ºC/1 min, extension at 72°C/2 min and denaturation at 95°C for 1 minute. Reactions were completed at 72°C/5 min. This produces a shorter PCR product of approximately 550 base pairs.

Template purification and sequencing

It is recommended to visualise the yield and purity of the amplification product using standard gel electrophoresis methods. The amplicon (approximately 700 base pairs for the W primer set, and 550 base pairs using the P locus) is purified using commercially available kits (eg. from Promega or Qiagen) to remove excess nucleotides, buffer and enzyme. After reconstituting in water the templates are ready for sequencing. For the W set the PCR primers work efficiently as sequencing primers. For the P set the additional sequencing primers are used.

MWG/Eurofins provide a commercial sequencing service. Details for sending the purified PCR product and sequencing primers are provided on their web site at https://ecom.mwgdna.com. Pre-paid 'value read' sequencing labels can be obtained in lots of 50, which has the advantage of allowing any number of samples to be sent over any time period without requiring separate invoices. Automation reduces costs and requires the tubes containing the template (7.5 µl of purified PCR product) to be labelled with one of the prepaid labels, which has a unique barcode. Also include separate tubes containing 4 µl of each forward and reverse primer (2 pmol per µl) for each PCR product to be sequenced. Details of the template and sequencing primer are supplied by logging on to the 'value read page' prior to dispatch of the template and primers. Sequences with the details supplied are then returned by email, usually within 48 hours.

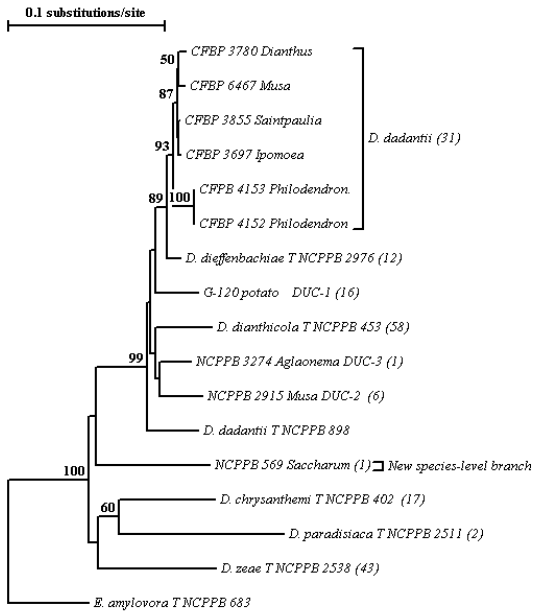
Phylogenetic analysis and reference sequence files

Alignments using Clustal and neighbour-joining phylogenies are produced using standard methods. The freely available MEGA phylogenetics package is most often used. Files containing previously sequenced reference sequences will be supplied which indicate positions for clipping to standard nucleotide start and finish positions.

References

Parkinson, N., Stead, D., Bew, J., Heeney, J. and Elphinstone, J. (2009) *Dickeya* species relatedness and clade structure determined by comparison of *recA* sequences. International Journal of Systematic and Evolutionary Microbiology 56; 2388-93

Waleron M, Waleron K, Podhajska A, Lojkowka E (2002) Genotyping of bacteria belonging to the former *Erwinia* genus by PCR-RFLP analyisis of a *recA* gene fragment. Microbiology 148; 583–595.



Dickeya phylogeny produced using the *recA* locus. Numbers in brackets indicate the number of strains from the population study that grouped in the clade.

3. *Xanthomonas spp.*.

The *gyrB* locus has been used to produce a phylogeny comprising all the species and pathovar type strains as well as a large number of poorly characterised strains (Parkinson *et al*., 2009). Seven clades were identified with long-branch lengths consistent with species-level taxa. One large clade comprises 60 pathovars with identical sequences, suggesting rapid pathovar evolution. There are two primer sets. We recommend that the 'standard' (S) primers are used initially and the alternative (A) set are used if strains are encountered which fail to produce a PCR product with the first set. An alternative primer set described by Young *et al*. (2008) amplifies a locus encompassing the region used in our reference set and provides an alternative procedure.

DNA purification.

We purify DNA from freshly grown colonies on yeast dextrose chalk agar plates. Chelex resin purification has proven to be reliable for all Gram-negative organisms.

Chelex 100 DNA preparation:

Resuspend 1.5 g Chelex 100 (BioRad 142-2832) in 25 ml H_2O (not TE buffer) to make a 6% solution, which can be used directly or stored after autoclaving. A 1ml pipette is used to aliquot the Chelex, which is maintained in suspension using a magnetic stirrer at moderate speed.

1) Prepare 1 ml cell suspensions to between OD_{650}, 0.1-0.2 and micro-centrifuge for 5 min at 10 000 x g.
2) Discard the supernatant carefully by using a pipette.
3) Resuspend the pellet in 300 µl of Chelex suspension (see above) by vortexing and incubate in a water bath at 56°C for 20 min.
4) Vortex at high speed for 10 seconds. Place the microfuge tube in a heated block at 100°C for 8 min. Note: Fix the caps using a weight to avoid the caps opening (explosively!) during heating.
5) Vortex tubes at high speed for 10 seconds, and immediately chill on ice.
6) Centrifuge the tube for 5 min at 14000 x g. Transfer 200 µl of the supernatant carefully to a new micro-centrifuge tube.
7) Use 2 µl aliquot of the supernatant as template DNA in a 50ul PCR reaction.

***gyrB* Primers**

Standard (S) gyrase PCR primers
XgyrPCR2F AAGCAGGGCAAGAGCGAGCTGTA
X.gyrrsp1 CAAGGTGCTGAAGATCTGGTC

Alternative (A) PCR primers
XgyrconpcrF1 AAGAGCGAGCTGTATCTGAAGGACGA
Xgyrconrpcr1 CGCGTCCTCGATGCGCACCTGCA

S- sequencing primers
X.gyr.fsp.s1 GGCAAGAGCGAGCTGTA
X.gyr.rsp3 CTGGTCGGCGGCCAC

A- sequencing primers
Xgyrconfsp1 GAGCTGTATCTGAAGGACGA
Xgyrconrsp1 CTCGATGCGCACCTGC

***gyrB* PCR amplification**.

A standard PCR reagent mix is used, final reagent concentrations were 2.5 mM $MgCl_2$, 300nM each primer, 0.2 mM dNTPs and 1 unit Taq polymerase in a final volume of 25 µl. Cycling parameters are:

Initial denaturation 94°C/2.5 min, followed by 34 cycles annealing at 50°C/45 sec, extension at 68°C/1 min and denaturation at 94°C/30 sec. Reactions were completed at 68°C/7 min. Although standard Taq is specified we have routinely used a commercially available Taq enzyme blended with a proof reading enzyme (Long PCR Enzyme mix; Fermentas).

Template purification and sequencing

It is recommended to visualise the yield and purity of the amplification product using standard gel electrophoresis methods. The amplicon (approximately 600 base pairs) is purified to remove excess nucleotides, buffer and enzyme using commercially available kits (eg. from Promega or Qiagen). After reconstituting in water the templates are ready for sequencing. Different sequencing primers are specified for the templates produced using the (S) and (A) PCR primers.

MWG/Eurofins provide a commercial sequencing service. Details for sending the purified PCR product and sequencing primers are provided on their web site at https://ecom.mwgdna.com. Pre-paid 'value read' sequencing labels can be obtained in lots of 50, which has the advantage of allowing any number of samples to be sent over any time period without requiring separate invoices. Automation reduces costs and requires the tubes containing the template (7.5 µl of purified PCR product) to be labelled with one of the prepaid labels, which has a unique barcode. Also include separate tubes containing 4 µl of each forward and reverse primer (2 pmol per µl) for each PCR product to be sequenced. Details of the template and sequencing primer are supplied by logging on to the 'value read page' prior to dispatch of the template and primers. Sequences with the details supplied are then returned by email, usually within 48 hours.

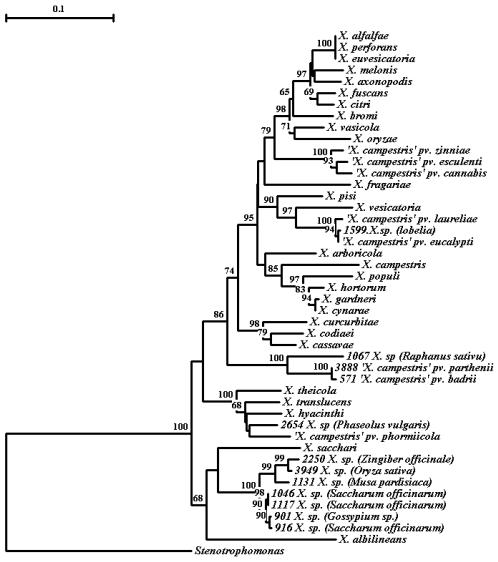
Phylogenetic analysis and reference sequence files

Alignments using Clustal and neighbour-joining phylogenies are produced using standard methods. The freely available MEGA phylogenetics package is most often used. Files containing previously sequenced reference sequences will be supplied which indicate positions for clipping to standard nucleotide start and finish positions.

References

Parkinson, N., Cowie, C., Heeney, J. and Stead, D. (2009) Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. IJSEM *59*, 264-274.

Young, J.M., Park, D.C., Shearman, H.M., Fargier,E. (2008) A multilocus sequence analysis of the genus *Xanthomonas* Syst. Appl. Microbiol. 31, 366-377



Xanthomonas gyrB phylogeny. Branches with species names are type strains. Branches and clades comprising strains with the plant hosts indicated in brackets are new 'species-level' clades.

4. *Erwinia amylovora*

PAVE	UMR Pathologie végétale (UA/INH/INRA) INRA centre Angers **PCR amplification of the *Erwinia amylovora recA* gene**	Date : 04/03/2010

1) Bacterial cultures

Strains were obtain from the Collection Française de Bactéries Phytopathogènes (CFBP), Beaucouzé, France.
Bacteria were grown on Luria-Bertani medium (LB) at 28°C for 24 h.

N°	Strain	Other strain designation	Host	Country	Year
1	CFBP1232	NCPPB 683, ATCC 15580, CCM 1114	*Pyrus communis*	United Kingdom	1959
2	CFBP7116		*Pyrus communis* cv. Angelys	France	2007
3	CFBP7130		*Pyrus communis*	Morocco	2007
4	CFBP4774		*Malus* sp. cv. Sans pareil	Lebanon	1998
5	CFBP3862		*Malus* X *domestica* var. Idared	Hungary	1996
6	CFBP3468		*Pyrus communis*	Turkey	1994
7	CFBP3066		*Pyrus* sp.	Greece	1987
8	CFBP3097		*Eryobotrya japonica*	Israel	1987
9	CFBP4637		*Pyrus communis*	Spain	1997
10	CFBP6449		*Cotoneaster salicifolius*	Austria	1993

2) DNA extraction

Wash the agar surface with 5 ml of sterile ultrapure water. Pipet out 1 ml of bacterial suspension and transfer it in a 1.5 ml sterile microtube. Boil the microtubes for 5 min and put them on ice. Centrifuge at 13000 g for 2 minutes. Store the supernatants (template DNA) in aliquots of 100µl at -20°C for late analysis.

3) PCR amplification

Amplification mix :

- 12.8 µl of ultrapure sterile water
- 4 µl of 5X GoTaq®Flexi buffer (Promega M8305)
- 1.2 µl of $MgCl_2$ (25mM) (Promega M8305)

- 0.5 µl of dNTP (2.5 mM each)
- 0.25 µl of recA2F (10µM)
- 0.25 µl of recA2R (10µM)
- 0.05 µl of GoTaq DNA Polymerase (5u/µl)
- Distribute 19 µl of amplification mix in a PCR reaction tube or well of PCR plate and add 1 µl of template DNA
- Close with caps and pulse briefly

Put the reaction tubes in the thermocycler and start the amplification program :

Thermal cycling conditions :

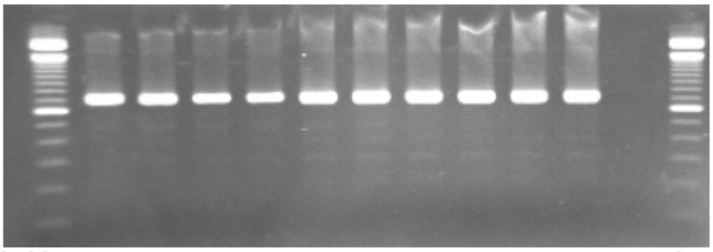
Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	45 s	30
Annealing	52	45 s	
Extension	72	1 min 30 s	
Final extension	72	10 min	1
Soak	15	indefinite	1

Primers :

recA2di	ATCTGGAATTCAGCCTG
recA2re	TCCATCATGCGCCTGGGTGAAGA

Size of the expected amplification product : 713 nt

4) Verification of DNA amplification



100pb Ladder
2% agarose gel in TBE buffer

5) Sequencing

The product is sequenced in both directions using a commercial sequencing service (Genoscreen, France) using forward and reverse recA2 primers.

ANNEX 3: SEQUENCE LIBRARIES

1. ***Dickeya* spp.**

Annex 3 Dickeya recA Euphresco fasta Feb2011.fas

2. ***Erwinia amylovora***

Annex 3 E amylovora recA Euphresco fasta Feb 2011.fas

3. ***Pseudomonas syringae complex***

Annex 3 P syringae complex rpoD Euphresco fasta Feb 2011.fas

4. ***Pseudonmonas syringae nuts and stone fruit isolates***

Annex 3 P syringae stone fruit rpoD Euphresco fasta Feb 2011.fas

5. ***Xanthomonas spp.***

Annex 3 Xanthomonas gyrB Euphresco 2011 fasta Feb 2011.fas