



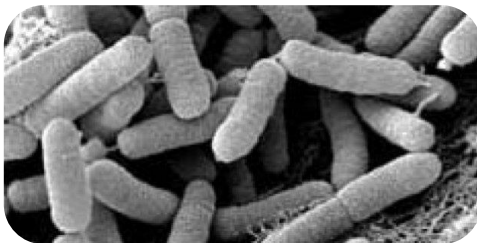
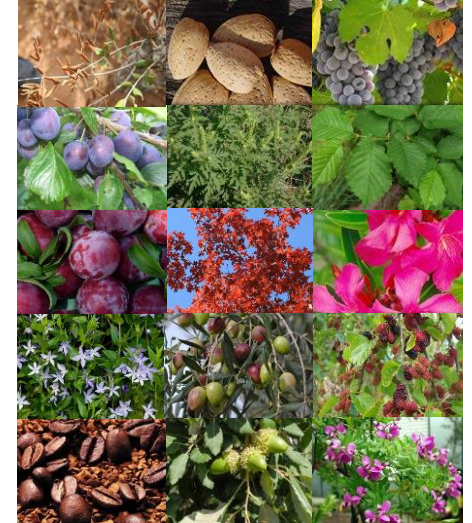
New tetraplex qPCR assays for simultaneous detection and identification of *Xylella fastidiosa* subspecies in plant tissues

Enora DUPAS, Martial BRIAND, Marie-Agnès JACQUES, Sophie CESBRON
PhD student IRHS, Inra, EMERSYS team

Background

Xylella fastidiosa

- Plant-associated bacteria
- In plant, limited to xylem vessels
- Transmission by insect vectors
- Large host range
 - Major social economic impact (olive tree, almond tree, citrus, grapevine...)
- Quarantine pathogen listed on A2 list of EPPO, directive 2000/29/CE revised in 2017

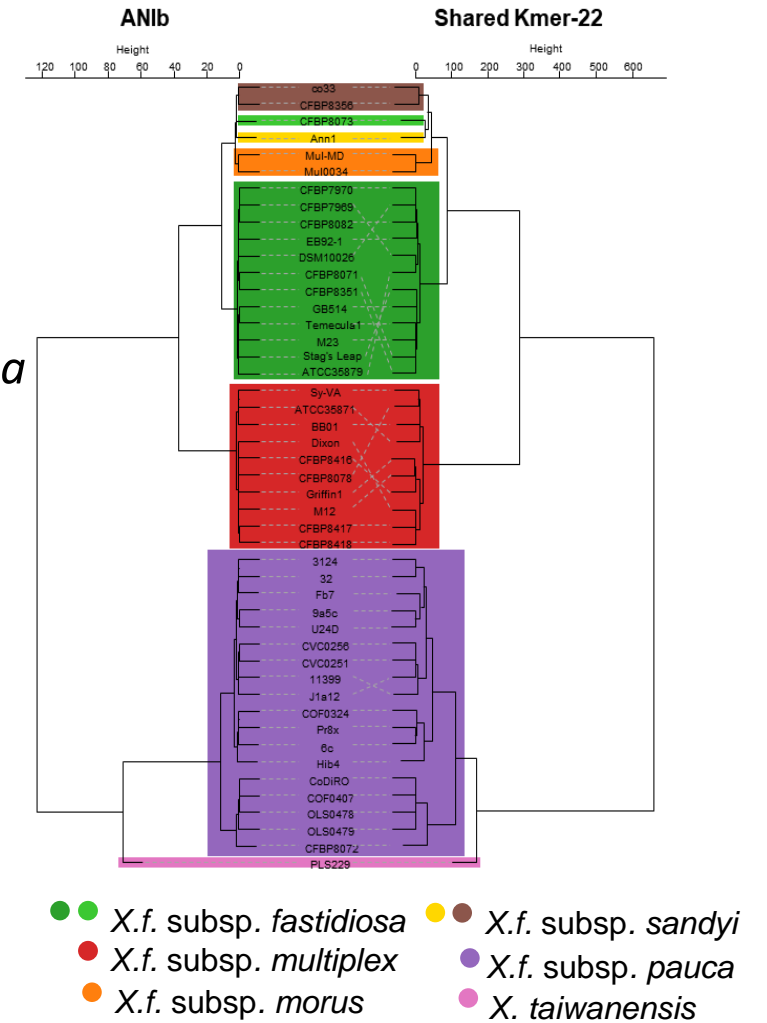


Background

Xylella fastidiosa

- Five subspecies described
 - X. fastidiosa* subsp. *fastidiosa* (Xff)
 - X. fastidiosa* subsp. *morus* (Xfmo)
 - X. fastidiosa* subsp. *sandyi* (Xfs)
 - X. fastidiosa* subsp. *multiplex* (Xfm)
 - X. fastidiosa* subsp. *pauca* (Xfp)
- One subsp. published (not supported)
 - X. fastidiosa* subsp. *tashke*

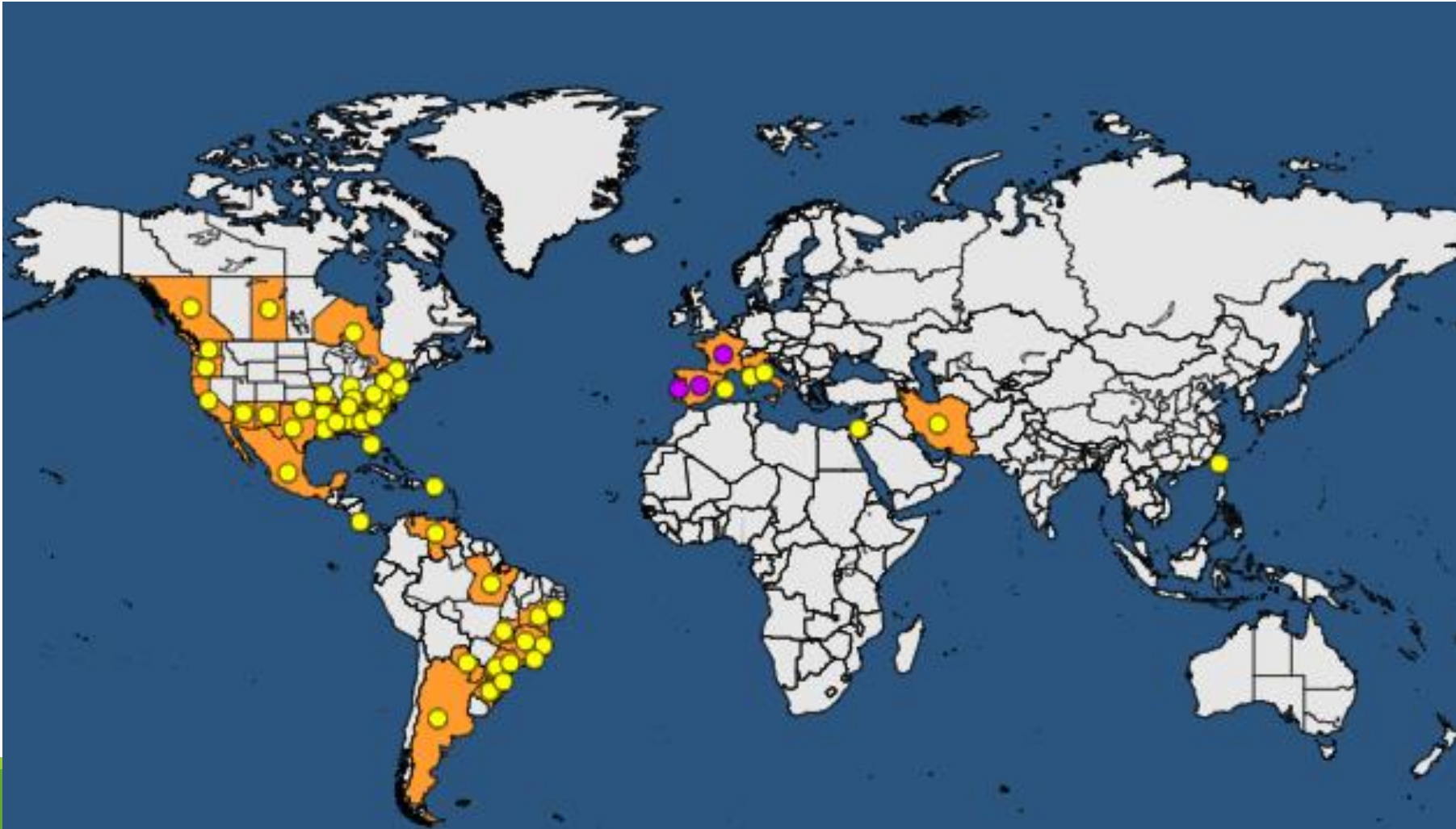
X. fastidiosa subsp. *fastidiosa*
sensu lato (Xffsl)



Denancé *et al.*, 2019

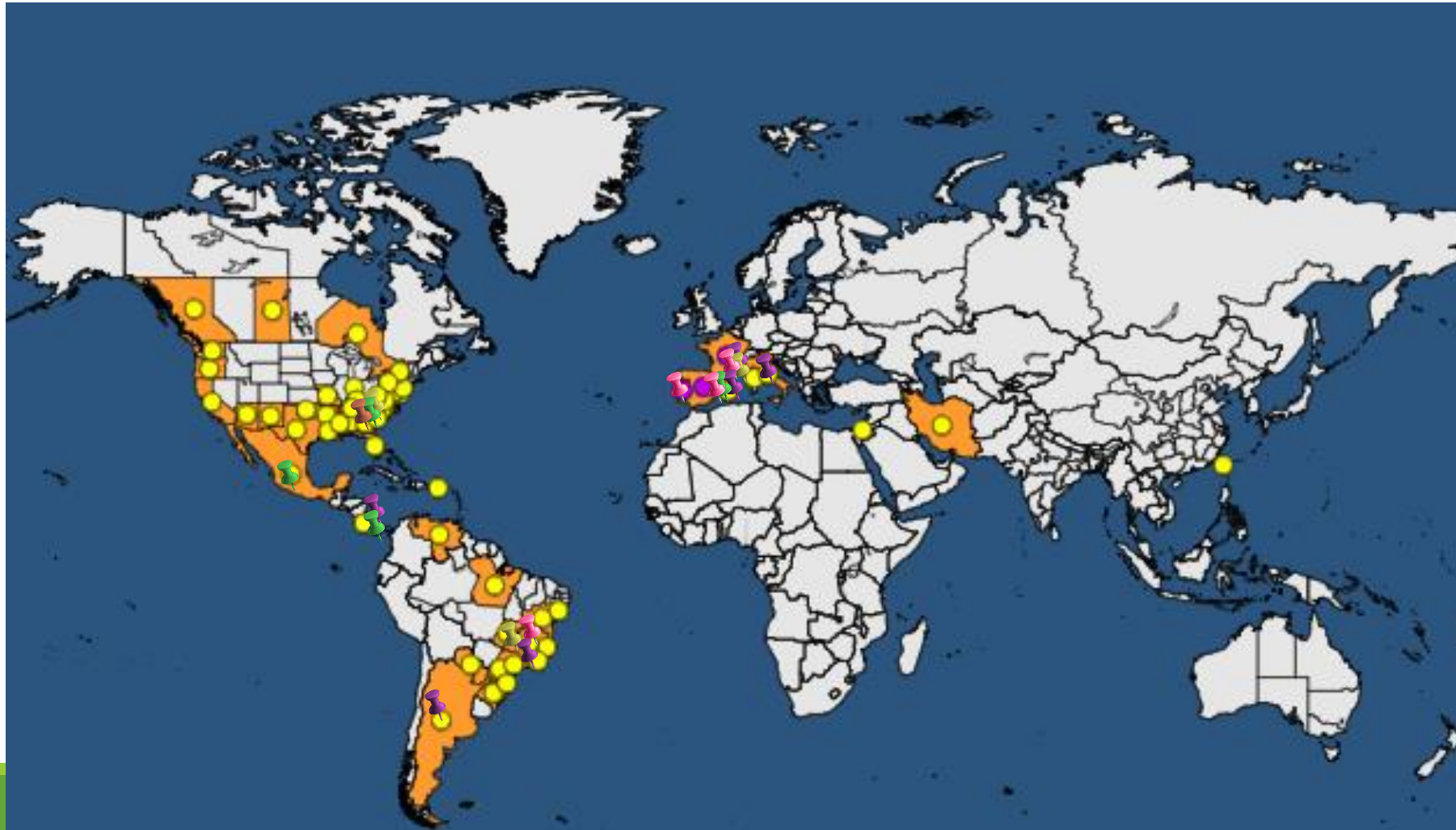
Background

- Geographical distribution of *Xylella* spp. (EFSA 2018)



Background

○ Geographical distribution of *Xylella* spp. (EFSA 2018)



Subspecies:

 *fastidiosa*

 *multiplex*

 *morus*

 *pauca*

 *sandyi*

Background

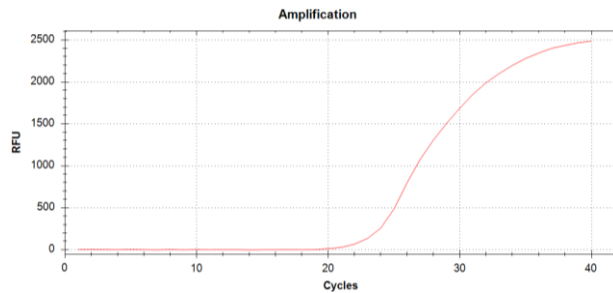
French surveillance plan

- Detection + Identification needed to set up plant management

1) Detection

Real-Time PCR (Harper *et al.*, 2010)

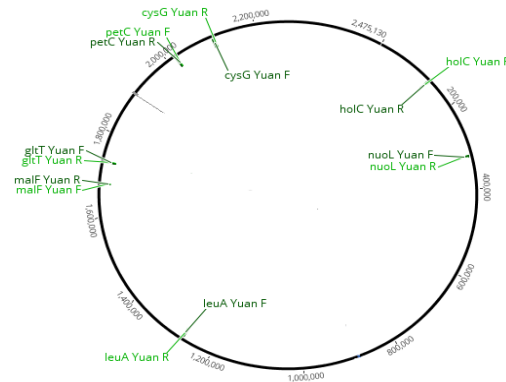
qPCR amplification



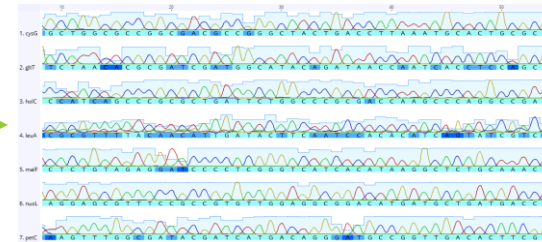
2) Subsp. identification

MLST (Yuan *et al.*, 2010)

PCR amplification of 7 housekeeping genes



Sequencing



Comparison to PubMLST database

ST	leuA	petC	malF	cysG	hoIC	nuoL	gltT
7	3	3	3	7	3	3	3

<https://pubmlst.org>

ST7 -> *Xylella fastidiosa* subsp. *multiplex*

Time	D0	D+0,5	D+1	D+4	D+5
Cost		0,52€	5,67€	35,7€	Total=41,89€/samples

Background

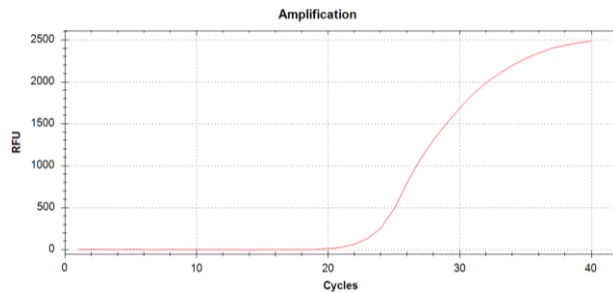
French surveillance plan

- Detection + Identification needed to set up plant management

1) Detection

Real-Time PCR (Harper *et al.*, 2010)

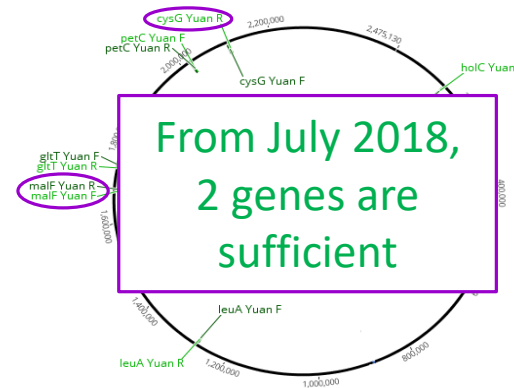
qPCR amplification



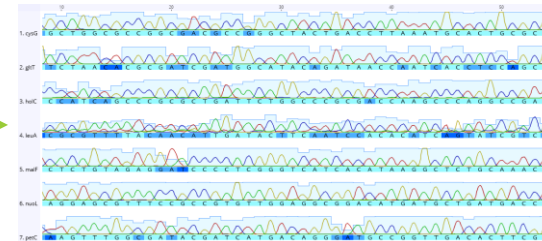
2) Subsp. identification

MLST (Yuan *et al.*, 2010)

PCR amplification of
7 housekeeping genes



Sequencing



Comparison to PubMLST
database

ST	leuA	petC	malF	cysG	holC	nuoL	gltT
7	3	3	3	7	3	3	3

<https://pubmlst.org>

ST7 -> *Xylella fastidiosa* subsp. *multiplex*

Time D0

D+0,5

D+1

D+4

D+5

Cost

0,52€

5,67€

35,7€

Total=41,89€/samples
(reduced scheme=12,35€/samples)

Background

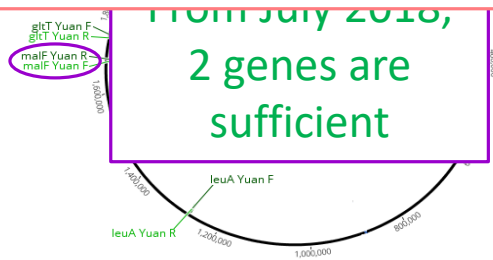
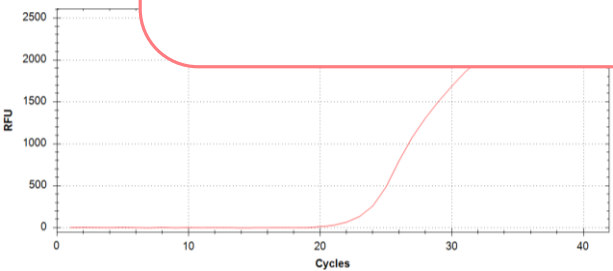
French surveillance plan

- Detection + Identification needed to set up plant management

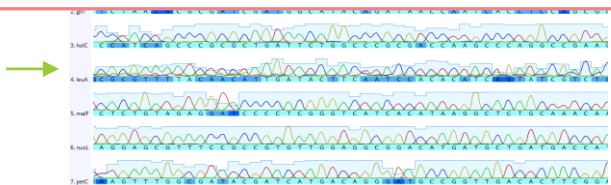
1) Detection

2) Subsp. identification

Time consuming and costly



From July 2018,
2 genes are
sufficient



ST	leuA	petC	malF	cysG	hoIC	nuoL	gltT
7	3	3	3	7	3	3	3

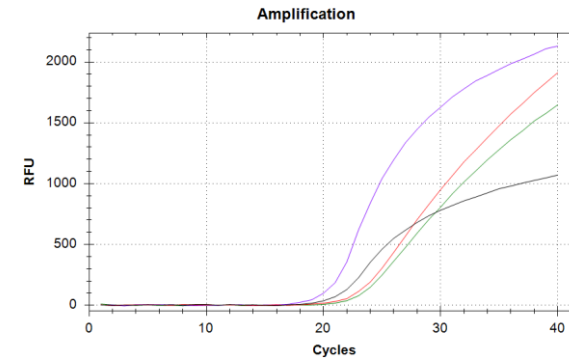
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ST7 -> *Xylella fastidiosa* subsp. *multiplex*

Time	D0	D+0,5	D+1	D+4	D+5
Cost		0,52€	5,67€	35,7€	Total=41,89€/samples (reduced scheme=12,35€/samples)

Objective

Development of multiplex qPCR assays for the detection/identification of *Xylella fastidiosa* subspecies



qPCR Assay development

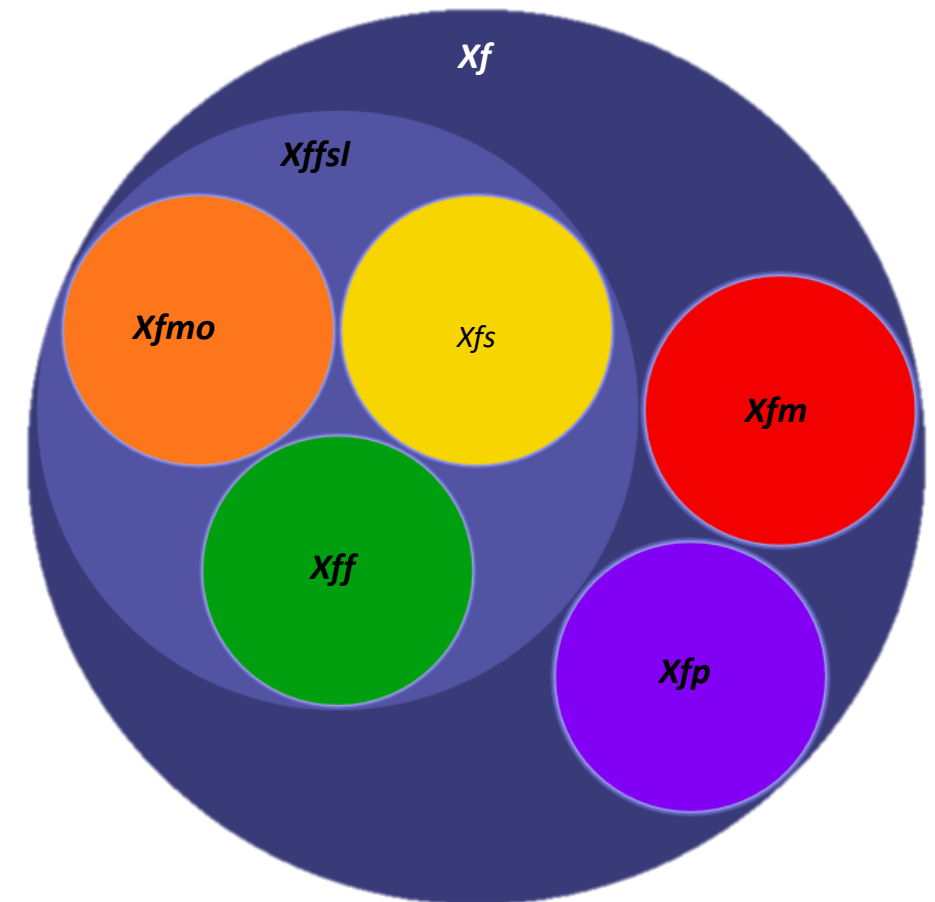
✓ Specific sequence search

- Using 47 genome sequences of *X. fastidiosa* with Sklf tool

✓ Design of primers and probes

- Using Primer3
- 6 primers/probes sets (*Xf*, *Xff*, *Xffsl*, *Xfm*, *Xfmo*, *Xfp*)

	Primers design criteria	Probes design criteria
Tm	60°C	70°C
Size	20 pb	20-26 pb
GC %	50%	50%



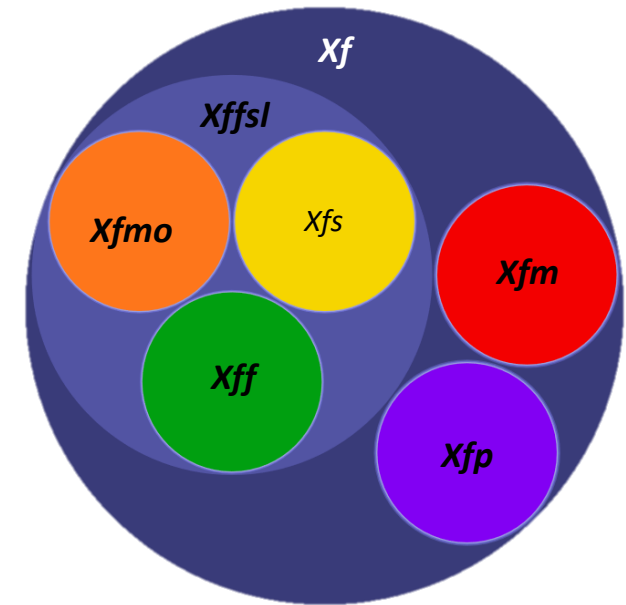
qPCR Assay development

✓ Specific sequence search

✓ Design of primers and probes

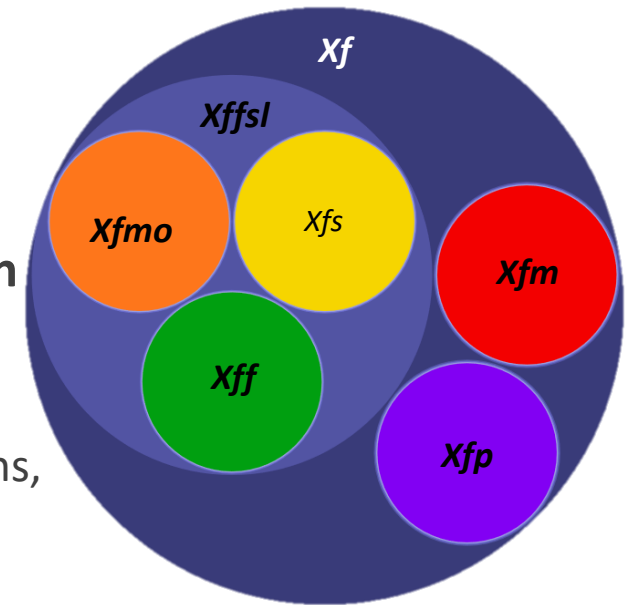
✓ Tests in silico

- 216 genomes available in EmerSys team
 - *Acidovorax*, *Burkholderia*, *Clavibacter*, *Curtobacterium*, *Dickeya*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Ralstonia*, *Xanthomonas*, *Xylella* and *Xylophilus*
- 154,478 bacterial Whole Genome Shotgun (available NCBI 22/08/2018)



qPCR Assay development

- ✓ Specific sequence search
- ✓ Design of primers and probes
- ✓ Tests in silico
- ✓ Evaluation of performance criteria in simplex qPCR assay on extracted DNA or bacterial suspensions
 - Detection threshold ($1\text{ng}.\mu\text{L}^{-1} - 1\text{fg}.\mu\text{L}^{-1}$)
 - Inclusivity (10 *Xff* strains, 1 *Xfmo* strains, 16 *Xfm* strains, 7 *Xfp* strains, 5 *Xfs* strains)
 - Exclusivity (39 *Xf* strains + 30 non-target strains)



Simplex qPCR Assay results



1 mono-copy target:
 $1 \text{ cp.mL}^{-1} = 1 \text{ cell.mL}^{-1}$

✅ Evaluation of performance criteria in simplex qPCR assay

✅ Detection threshold ($1 \text{ ng.}\mu\text{L}^{-1} - 1 \text{ fg.}\mu\text{L}^{-1}$)

Subspecies amplified	New qPCR		qPCR Harper et al., 2010
	Primers used	Detection threshold	
CFBP 7970 (Xff)	XF	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})	$4.10^4 \text{ cp.mL}^{-1}$ (100 pg.mL^{-1})
CFBP 7970 (Xff)	XFF	$4.10^4 \text{ cp.mL}^{-1}$ (100 pg.mL^{-1})	$4.10^4 \text{ cp.mL}^{-1}$ (100 pg.mL^{-1})
CFBP 7970 (Xff)	XFFSL	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})	$4.10^4 \text{ cp.mL}^{-1}$ (100 pg.mL^{-1})
CFBP 8416 (Xfm)	XFM	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})	$4.10^4 \text{ cp.mL}^{-1}$ (100 pg.mL^{-1})
CFBP 8084 (Xfmo)	XFMO	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})
CFBP 8402 (Xfp)	XFP	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})	$4.10^3 \text{ cp.mL}^{-1}$ (10 pg.mL^{-1})

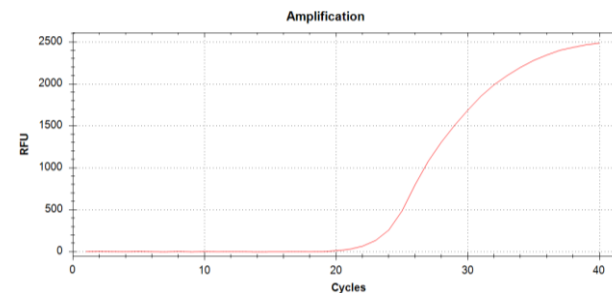
Simplex qPCR Assay results

✔ Evaluation of performance criteria in simplex qPCR assay

✔ Detection threshold ($1\text{ng}.\mu\text{L}^{-1}$ – $1\text{fg}.\mu\text{L}^{-1}$)

✔ Inclusivity (target strains): 100%

- (10 *Xff* strains, 1 *Xfmo* strains, 16 *Xfm* strains, 7 *Xfp* strains, 5 *Xfs* strains)
- **Primers and probes always amplified the subspecies for which they were designed.**



Simplex qPCR Assay results

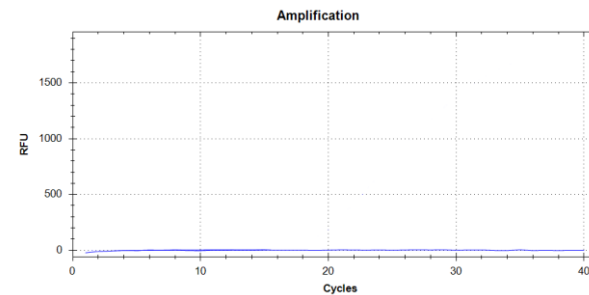
✔ Evaluation of performance criteria in simplex qPCR assay

✔ Detection threshold ($1\text{ng}\cdot\mu\text{L}^{-1} - 1\text{fg}\cdot\mu\text{L}^{-1}$)

✔ Inclusivity (10 *Xff* strains, 1 *Xfmo* strains, 15 *Xfm* strains, 7 *Xfp* strains, 1 *Xfs* strains)

✔ Exclusivity (non target strains): 100%

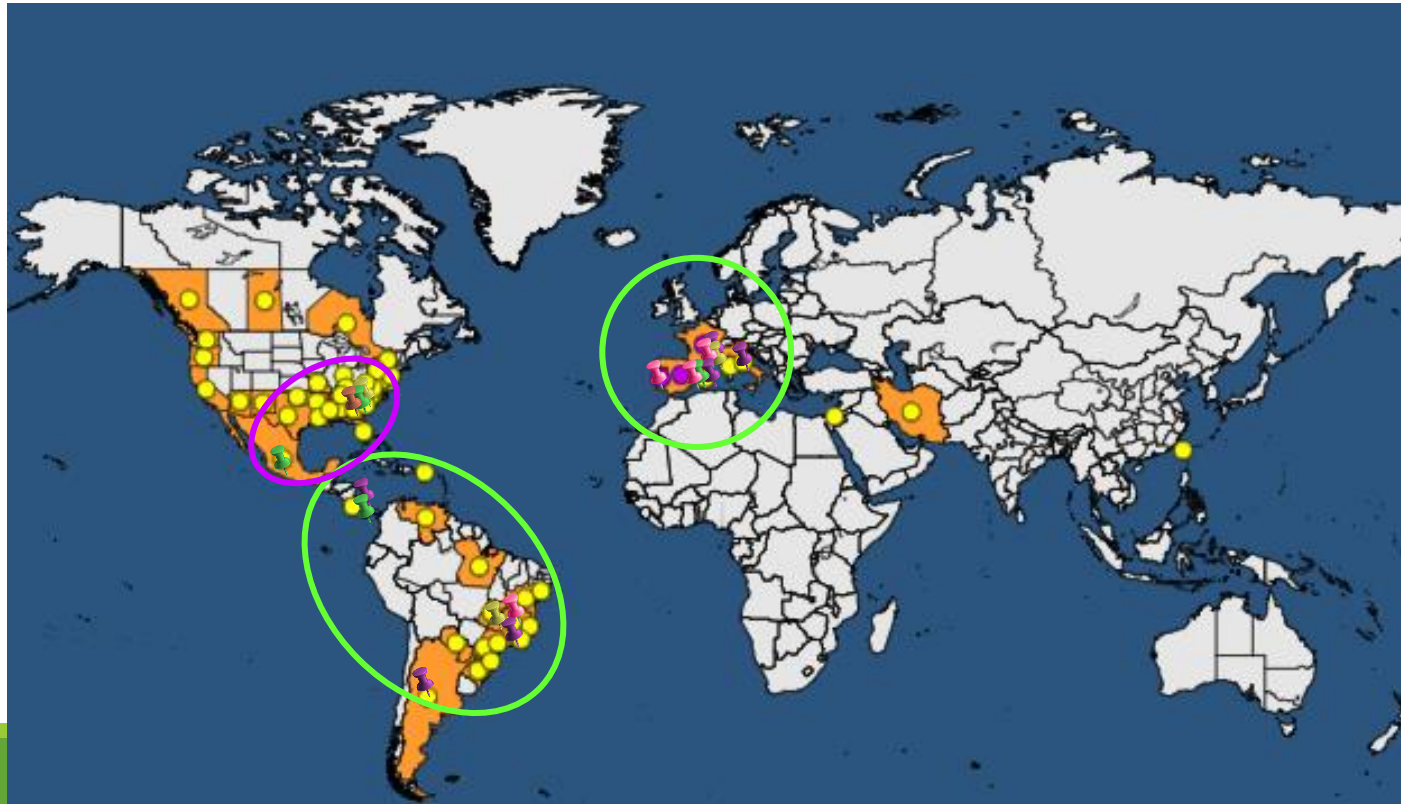
- 39 *Xf* strains + 30 non target strains (*Agrobacterium*, *Clavibacter*, *Dickeya*, *Ensifer*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Rhizobium*, *Stenotrophomonas*, *Xanthomonas*, *Xylophilus*)
- None of the primers and probes amplified a subspecies that they were not designed for.



Multiplex qPCR Assay development

Multiplexing of primers and probe to amplify:

- XF + XFF, XFM, XFP (European and Central - South American subsp.)
- XF + XFF, XFM, XFMO (North American subsp.)
- XF + XFFSL, XFM, XFP (All *Xylella fastidiosa* diversity)



Subspecies

-  *fastidiosa*
-  *multiplex*
-  *morus*
-  *pauca*
-  *sandyi*

Multiplex qPCR Assay results



1 mono-copy target:
 $1 \text{ cp.mL}^{-1} = 1 \text{ cell.mL}^{-1}$

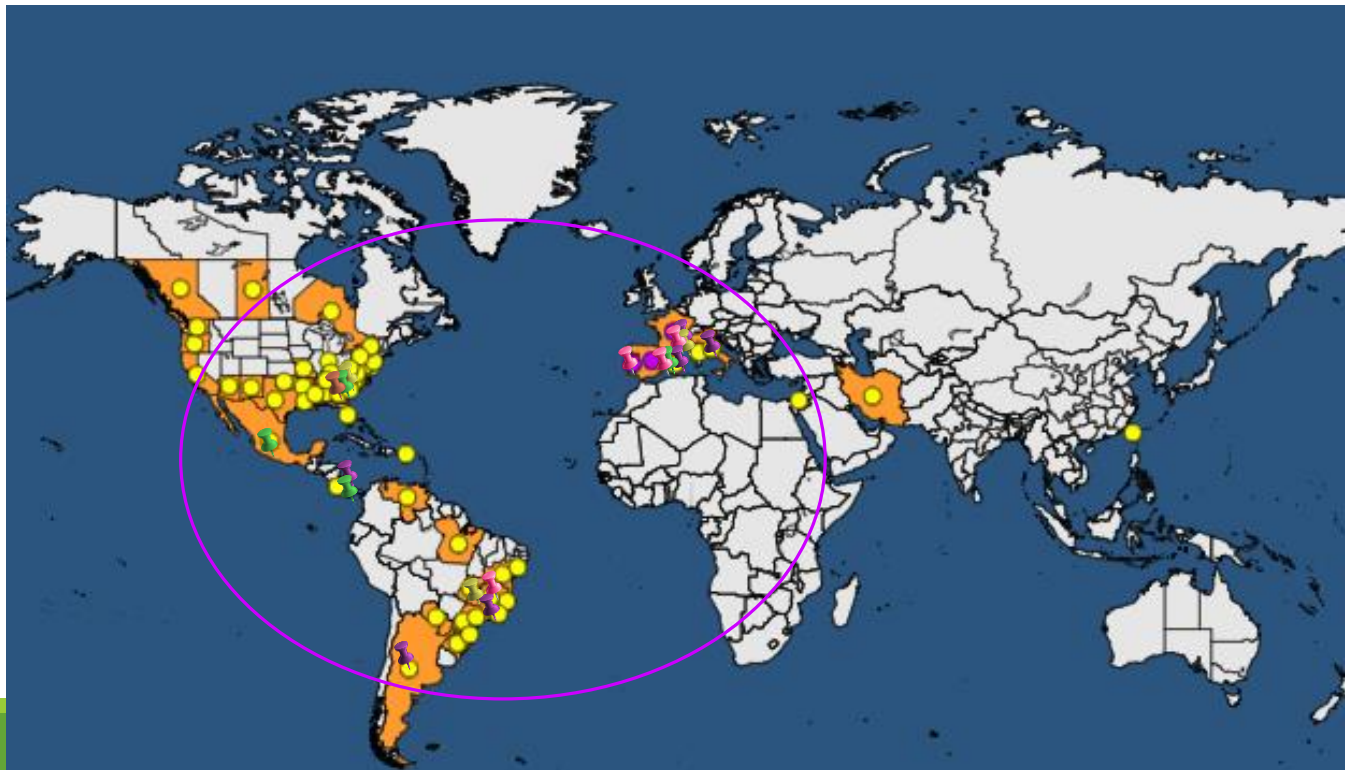
- Evaluation of performance criteria in multiplex qPCR assay
- Detection threshold ($1 \text{ ng.}\mu\text{L}^{-1} - 1 \text{ fg.}\mu\text{L}^{-1}$)

New qPCR		qPCR Harper <i>et al.</i> , 2010
Primers used	Detection threshold	
XF, XFF + XFM + XFMO	4.10^3 to $4.10^4 \text{ cp.mL}^{-1}$ (10 - 100 pg.mL^{-1})	4.10^3 to $4.10^4 \text{ cp.mL}^{-1}$ (10 - 100 pg.mL^{-1})
XF, XFF + XFM + XFP	4.10^3 to $4.10^4 \text{ cp.mL}^{-1}$ (10 - 100 pg.mL^{-1})	4.10^3 to $4.10^4 \text{ cp.mL}^{-1}$ (10 - 100 pg.mL^{-1})
XF, XFFSL + XFM + XFP	$4.10^4 \text{ cp.mL}^{-1}$ (100 pg.mL^{-1})	4.10^3 to $4.10^4 \text{ cp.mL}^{-1}$ (10 - 100 pg.mL^{-1})

Multiplex qPCR Assay development

Multiplexing of primers and probe to amplify:

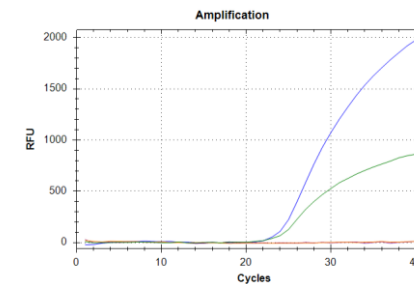
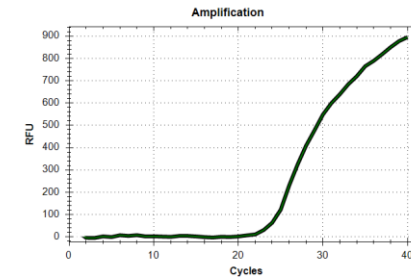
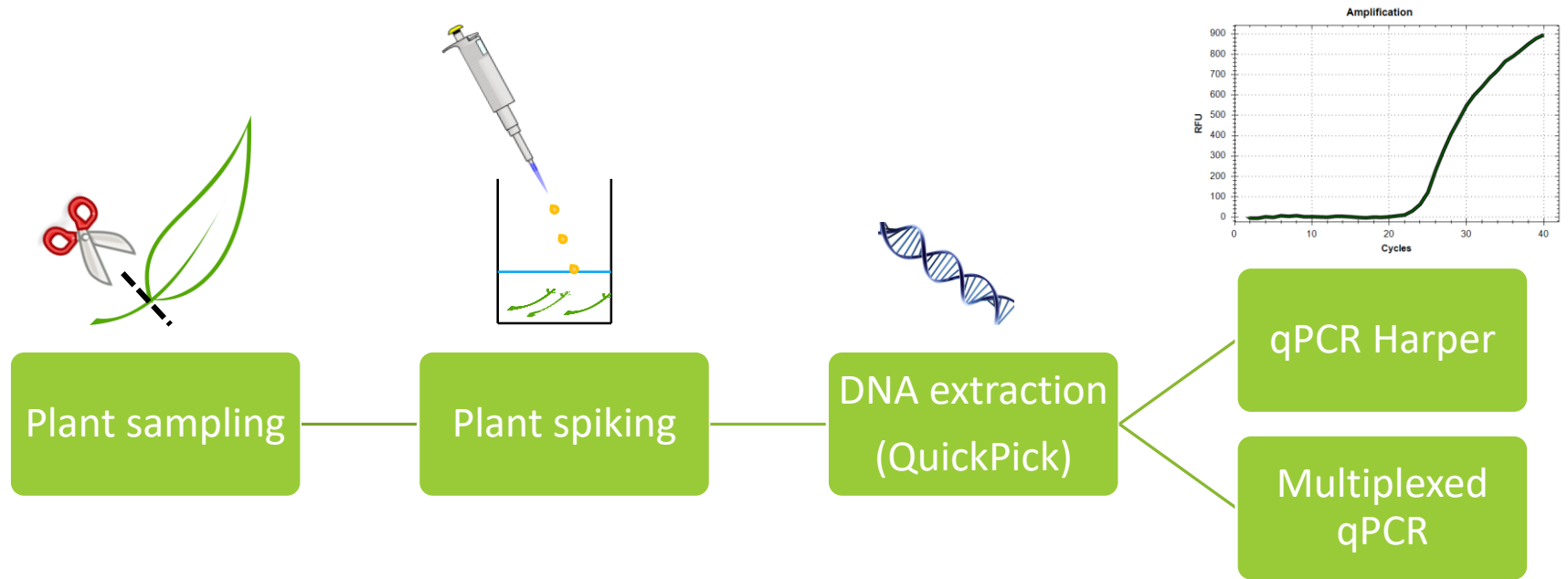
- XF + XFF, XFM, XFP (European and Central - South American subsp.)
- XF + XFF, XFM, XFMO (North American subsp.)
- XF + XFFSL, XFM, XFP (All *Xylella fastidiosa* diversity)



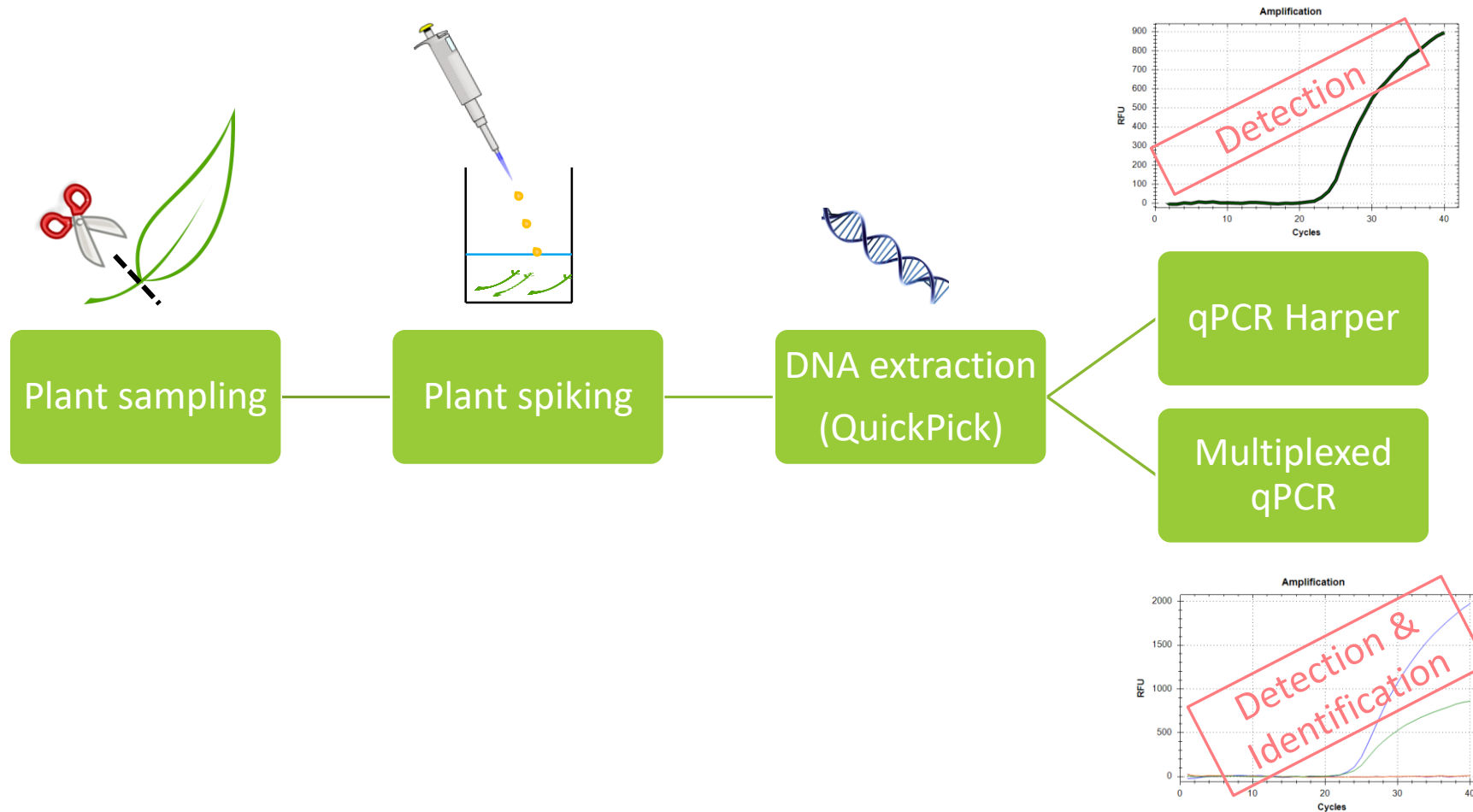
Subspecies:

-  *fastidiosa*
-  *multiplex*
-  *morus*
-  *pauca*
-  *sandyi*

qPCR multiplex protocol











qPCR multiplex protocol



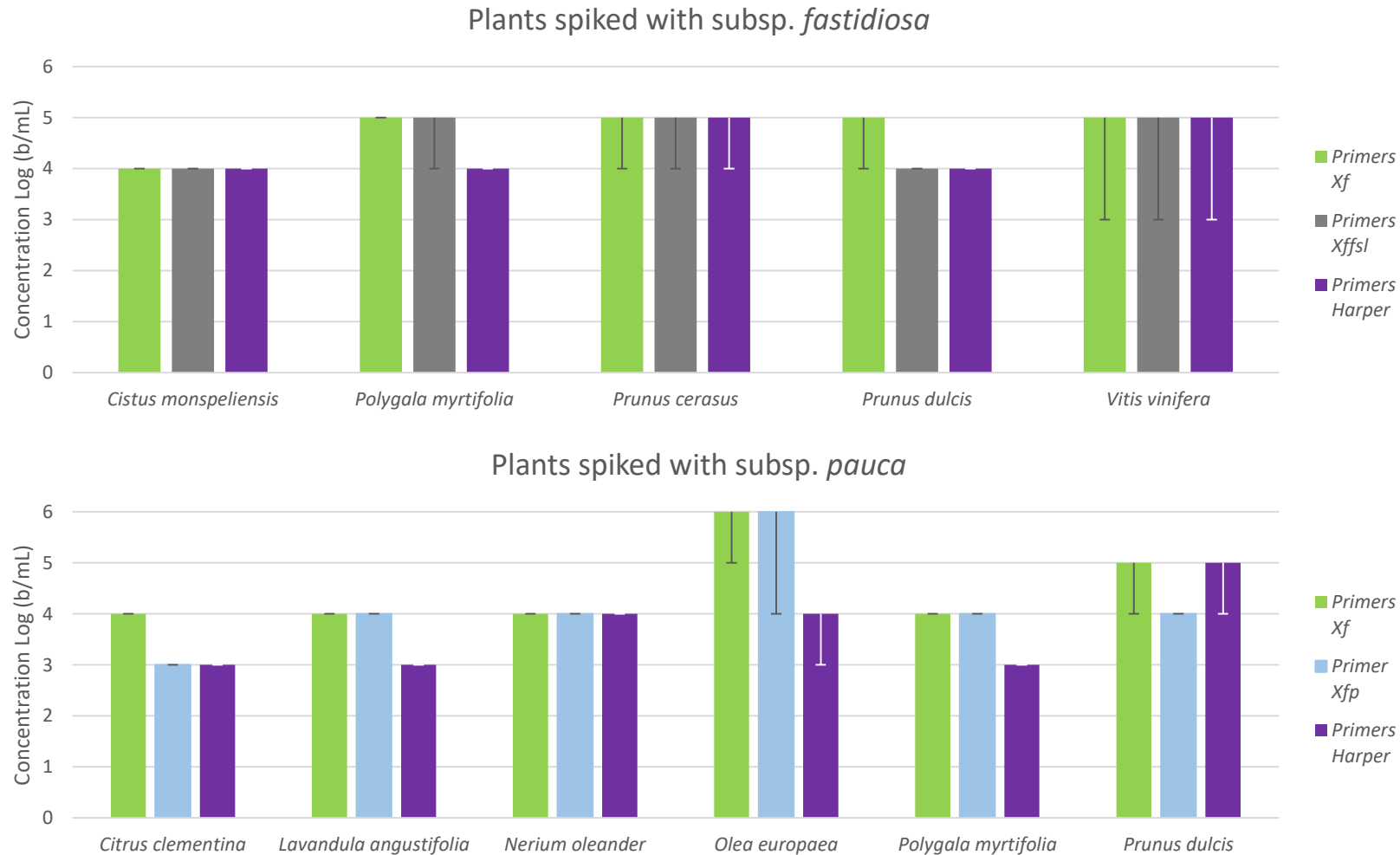
qPCR multiplex protocol

- 13 matrices were spiked with different subspecies according to natural identification

<p><i>Citrus clementin</i></p>  <p>Xfm Xfp</p>	<p><i>Cistus monspeliensi</i></p>  <p>Xff Xfm</p>	<p><i>Helichrysum italicum</i></p>  <p>Xfm</p>	<p><i>Lavandula</i></p>  <p>Xfm Xfp</p>	<p><i>Nerium oleander</i></p>  <p>Xfm Xfp Xfm + Xfp</p>	<p><i>Olea europaea</i></p>  <p>Xfm Xfp Xfm + Xfp</p>	
<p><i>Polygala</i></p>  <p>Xff Xfm Xfmo Xfp Xff + Xfm + Xfp</p>	<p><i>Prunus cerasus</i></p>  <p>Xff Xfm</p>	<p><i>Prunus dulcis</i></p>  <p>Xff Xfm Xfp Xff + Xfm + Xfp</p>	<p><i>Quercus ilex</i></p>  <p>Xfm</p>	<p><i>Quercus robur</i></p>  <p>Xfm</p>	<p><i>Rosmarinus officinalis</i></p>  <p>Xfm</p>	<p><i>Vitis vinifera</i></p>  <p>Xff Xfm</p>

Results:

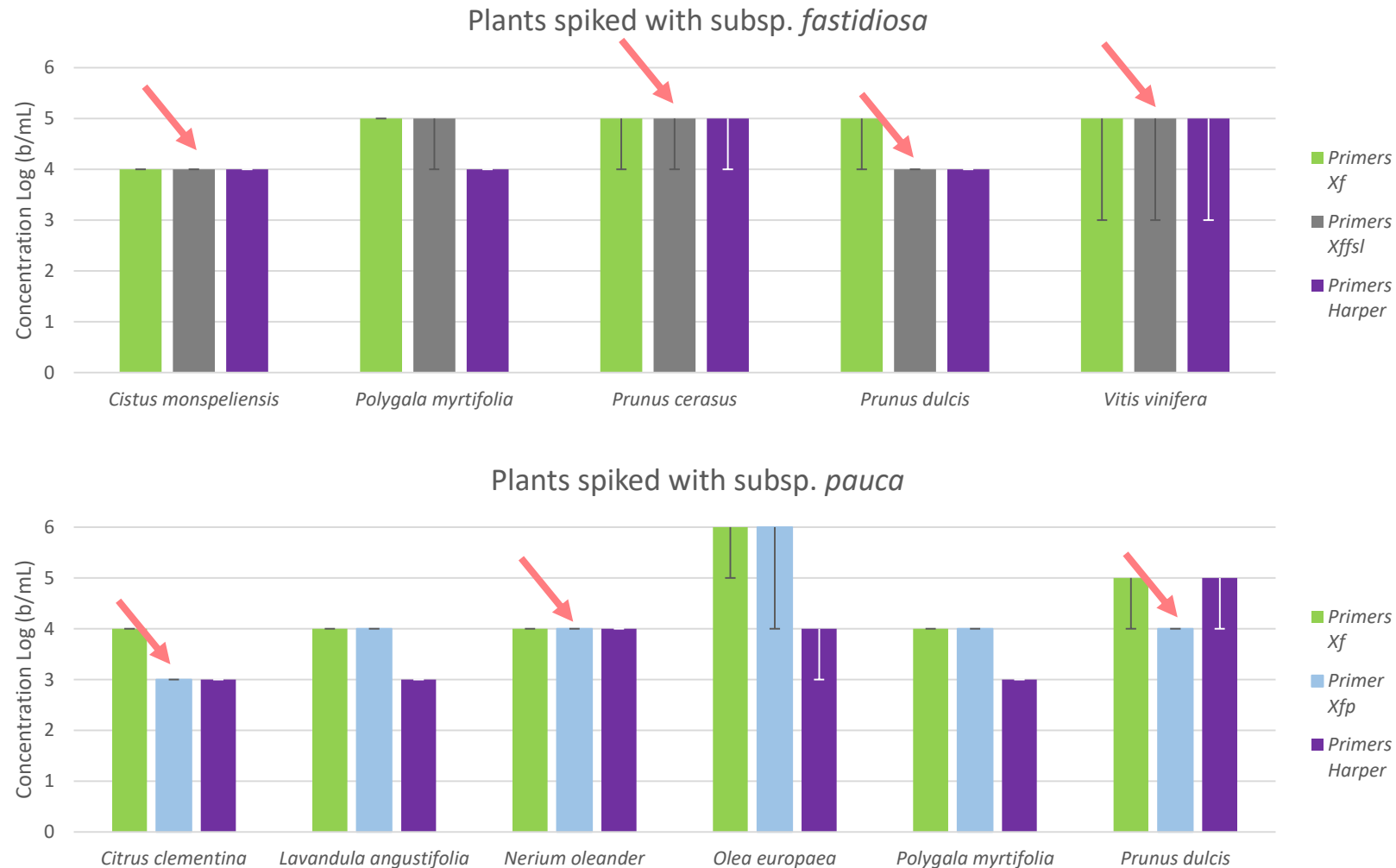
Detection threshold of spiked plants with multiplex qPCR assays



- Detection of *Xf* and identification of its subsp. in all tested plants

Results:

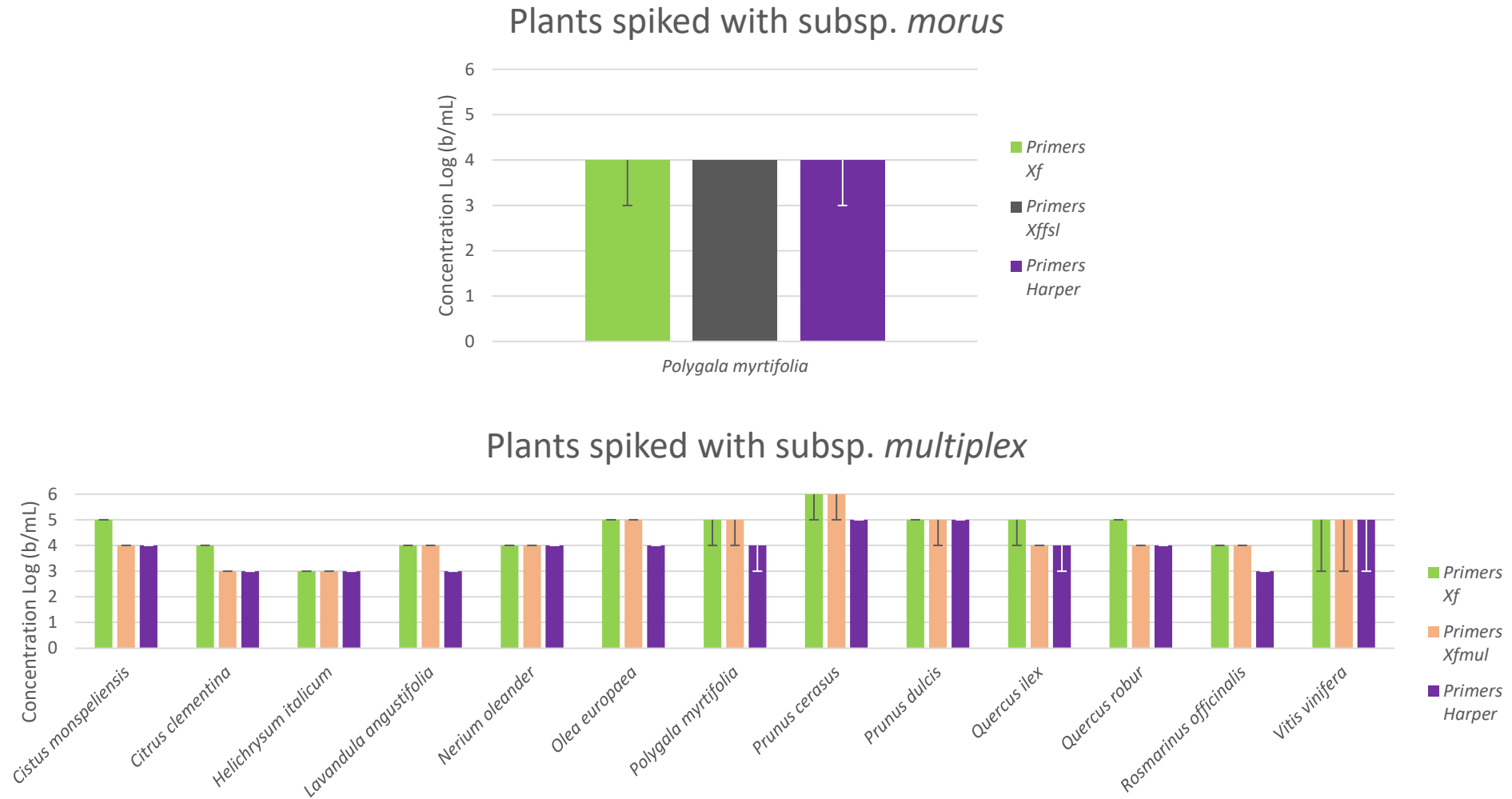
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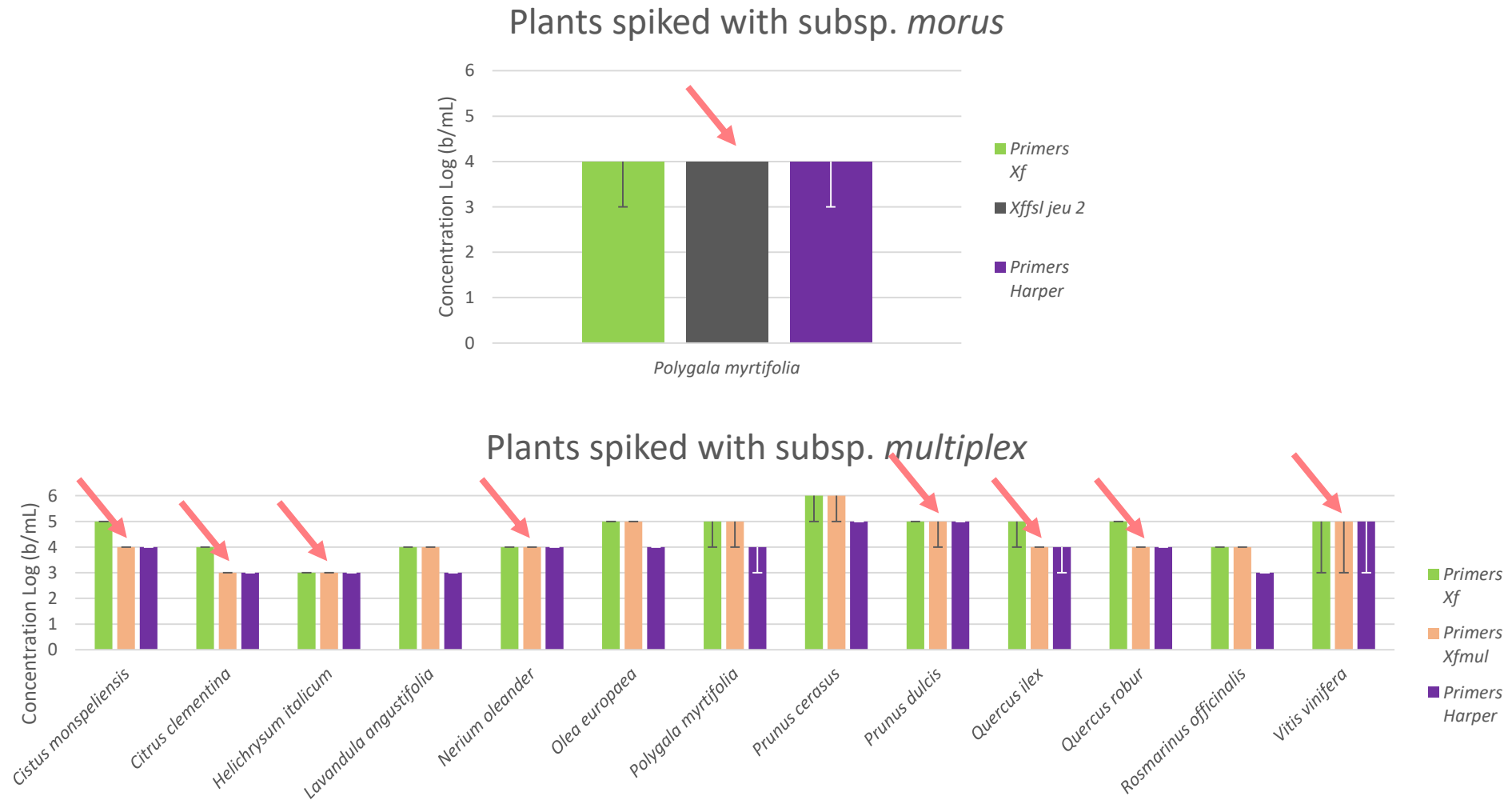
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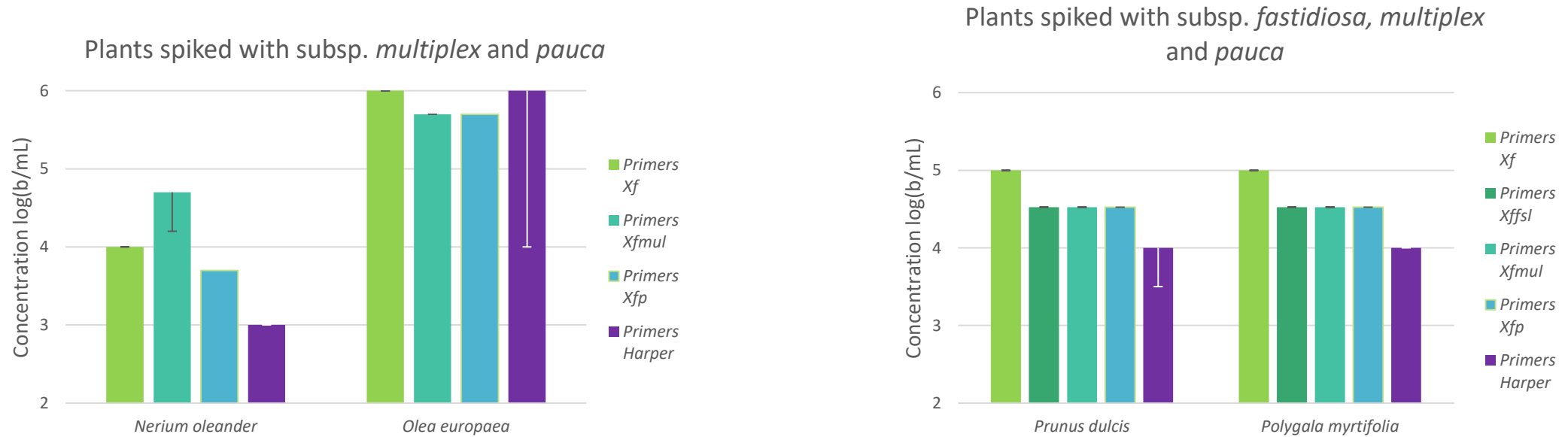
Detection threshold of spiked plants with multiplex qPCR assays



- Detection of *Xf* and identification of its subsp. in all tested plants

Results:

Detection threshold of spiked plants with a mix of 2 or 3 strains



- Detection of *Xf* and identification of its different subspecies in *Nerium oleander*, *Olea europaea*, *Prunus dulcis* and *Polygala myrtifolia*

Results:

Detection threshold of naturally infected samples

n°	Host plant	Place (year)	XF Average Ct ± SEM	XFFSL Average Ct ± SEM	XFM Average Ct ± SEM	Harper et al., 2010 Average Ct± SEM	MLST subspecies identification
1	<i>Asparagus acutifolius</i>	Aleria, France (2017)	-	-	-	+	unknown
2	Caprifoliaceae	Bonifaccio, France (2017)	+	+	-	+	unknown
3	<i>Cistus monspeliensis</i>	Ajaccio, France (2018)	-	-	-	+	unknown
4	<i>Helichrysum italicum</i>	Propriano, France (2017)	+	-	+	+	unknown
5	<i>Lavandula stoechas</i>	Vignola, France (2017)	+	-	+	+	unknown
6	<i>Lavandula stoechas</i>	Propriano, France (2017)	-	-	-	+	unknown
7	<i>Myrtus communis</i>	Ajaccio, France (2018)	-	-	-	+	unknown
8	<i>Olea europaea</i>	Afa, France (2017)	-	-	+	+	unknown
9	<i>Olea europaea</i>	Bonifaccio, France (2018)	-	-	-	+	unknown
10	<i>Olea europaea</i>	Vignola, France (2017)	+	+	-	+	unknown
11	<i>Phylirea angustifolia</i>	Bonifaccio, France (2017)	-	+	-	+	unknown
12	<i>Polygala myrtifolia</i>	Vignola, France (2017)	+	-	+	+	Suspected Xfm
13	<i>Polygala myrtifolia</i>	Porto-Vecchio, France (2018)	+	-	+	+	unknown
14	<i>Quercus ilex</i>	Ostriconi, France (2018)	-	-	-	+	unknown
15	<i>Quercus suber</i>	Porticcio, France (2018)	-	-	-	+	unknown
16	<i>Spartium junceum</i>	Corbara, France (2017)	+	-	+	+	unknown

Results:

Detection threshold of naturally infected samples

n°	Host plant	Place (year)	XF Average Ct ± SEM	XFFSL Average Ct ± SEM	XFM Average Ct ± SEM	Harper et al., 2010 Average Ct± SEM	MLST subspecies identification
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2	Caprifoliaceae	Bonifaccio, France (2017)	+	+	-	+	unknown
3	<i>Cistus monspeliensis</i>	Ajaccio, France (2018)	-	-	-	+	unknown
4	Helichrysum italicum	Propriano, France (2017)	+	-	+	+	unknown
5	Lavandula stoechas	Vignola, France (2017)	+	-	+	+	unknown
6	<i>Lavandula stoechas</i>	Propriano, France (2017)	-	-	-	+	unknown
7	<i>Myrtus communis</i>	Ajaccio, France (2018)	-	-	-	+	unknown
8	<i>Olea europaea</i>	Afa, France (2017)	-	-	+	+	unknown
9	<i>Olea europaea</i>	Bonifaccio, France (2018)	-	-	-	+	unknown
10	Olea europaea	Vignola, France (2017)	+	+	-	+	unknown
11	<i>Phyllirea angustifolia</i>	Bonifaccio, France (2017)	-	+	-	+	unknown
12	Polygala myrtifolia	Vignola, France (2017)	+	-	+	+	Suspected Xfm
13	Polygala myrtifolia	Porto-Vecchio, France (2018)	+	-	+	+	unknown
14	<i>Quercus ilex</i>	Ostriconi, France (2018)	-	-	-	+	unknown
15	<i>Quercus suber</i>	Porticcio, France (2018)	-	-	-	+	unknown
16	Spartium junceum	Corbara, France (2017)	+	-	+	+	unknown

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3	<i>Cistus monspeliensis</i>	Ajaccio, France (2018)	-	-	-	+	unknown
4	Helichrysum italicum	Propriano, France (2017)	+	-	+	+	unknown
5	Lavandula stoechas	Vignola, France (2017)	+	-	+	+	unknown
6	<i>Lavandula stoechas</i>	Propriano, France (2017)	-	-	-	+	unknown
7	<i>Myrtus communis</i>	Ajaccio, France (2018)	-	-	-	+	unknown
8	<i>Olea europaea</i>	Afa, France (2017)	-	-	+	+	unknown
9	<i>Olea europaea</i>	Bonifaccio, France (2018)	-	-	-	+	unknown
10	Olea europaea	Vignola, France (2017)	+	+	-	+	unknown
11	<i>Phyllirea angustifolia</i>	Bonifaccio, France (2017)	-	+	-	+	unknown
12	Polygala myrtifolia	Vignola, France (2017)	+	-	+	+	Suspected Xfm
13	Polygala myrtifolia	Porto-Vecchio, France (2018)	+	-	+	+	unknown
14	<i>Quercus ilex</i>	Ostriconi, France (2018)	-	-	-	+	unknown
15	<i>Quercus suber</i>	Porticcio, France (2018)	-	-	-	+	unknown
16	Spartium junceum	Corbara, France (2017)	+	-	+	+	unknown

Results:

Detection threshold of naturally infected samples

n°	Host plant	Place (year)	XF Average Ct ± SEM	XFFSL Average Ct ± SEM	XFM Average Ct ± SEM	Harper et al., 2010 Average Ct± SEM	MLST subspecies identification
1	<i>Asparagus acutifolius</i>	Aleria, France (2017)	-	-	-	37.60 ± 0.00	unknown
2	<i>Caprifoliaceae</i>	Bonifaccio, France (2017)	27.86 ± 0.00	33.67 ± 1.42	-	34.97 ± 0.53	unknown
3	<i>Cistus monspeliensis</i>	Ajaccio, France (2018)	-	-	-	36.52 ± 0.59	unknown
4	<i>Helichrysum italicum</i>	Propriano, France (2017)	27.35 ± 0.67	-	27.25 ± 0.23	30.85 ± 0.04	unknown
5	<i>Lavandula stoechas</i>	Vignola, France (2017)	30.75 ± 0.73	-	26.27 ± 0.38	29.50 ± 0.13	unknown
6	<i>Lavandula stoechas</i>	Propriano, France (2017)	-	-	-	34.81 ± 1.40	unknown
7	<i>Myrtus communis</i>	Ajaccio, France (2018)	-	-	-	35.66 ± 0.52	unknown
8	<i>Olea europaea</i>	Afa, France (2017)	-	-	36.83 ± 0.00	34.01 ± 0.77	unknown
9	<i>Olea europaea</i>	Bonifaccio, France (2018)	-	-	-	37.68 ± 0.27	unknown
10	<i>Olea europaea</i>	Vignola, France (2017)	25.56 ± 0.00	29.91 ± 0.80	-	32.94 ± 0.18	unknown
11	<i>Phylirea angustifolia</i>	Bonifaccio, France (2017)	-	30.52 ± 0.21	-	33.99 ± 1.09	unknown
12	<i>Polygala myrtifolia</i>	Vignola, France (2017)	24.86 ± 0.04	-	25.00 ± 0.03	25.96 ± 0.04	Suspected Xfm
13	<i>Polygala myrtifolia</i>	Porto-Vecchio, France (2018)	30.14 ± 0.58	-	29.52 ± 0.17	32.82 ± 0.41	unknown
14	<i>Quercus ilex</i>	Ostriconi, France (2018)	-	-	-	36.65 ± 0.00	unknown
15	<i>Quercus suber</i>	Porticcio, France (2018)	-	-	-	35.94 ± 0.00	unknown
16	<i>Spartium junceum</i>	Corbara, France (2017)	23.68 ± 0.17	-	23.97 ± 0.14	24.97 ± 0.06	unknown

Take home message

- **Development of six sets** of primers and probes that are **subspecies-specific**
 - **Highly specific:** no cross-amplifications with any other subsp., species or genera
- qPCR assays usable in **tetraplex**
 - **Allows detection** of **unique** and **multiple subspecies infections**
- Detection threshold is **as good as Harper's qPCR** detection threshold on **pure cultures**
- Detection threshold **in plant matrices is equal or a little higher than Harper's qPCR** detection threshold
 - But still interesting because it provides the subspecies identification at levels where MLST doesn't work ($Ct \geq 30 \Rightarrow [bact] < 10^5 - 10^4 \text{ b.mL}^{-1}$)
- Cheaper and faster than the current identification scheme (0,37€/sample VS 12,35-41,89€/sample)
- Candidate for consideration as official method



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