Title **EuroBlight 12-plex SSR genotyping of *Phytophthora infestans***

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Background

The plant pathogen, *Phytophthora infestans* is the cause of potato and tomato late blight and results in significant crop losses at a global scale. The pathogen population is documented to reproduce asexually, resulting in the widespread dominance of well-adapted named clonal lineages such as US-1 or EU_13_A2. In regions where both A1 and A2 mating types co-exist, such as Mexico (Wang et al., 2017) and northeast Europe (Yuen and Andersson 2013), it may also form oospores which germinate to generate highly diverse sexually reproducing populations. Pathogen phenotypic or genotypic monitoring is important to understand population change and to help inform disease management practices. Genotyping has relied on a diverse range of molecular methods (Cooke and Lees, 2004). Simple Sequence Repeat (SSR) markers (also known as microsatellites) have proven valuable for tracking pathogen population change for a range of reasons. The methods are more robust than previous methods such as RFLPs as they are directly amplified from relatively impure DNA extractions (including mixes of pathogen and host DNA) and both alleles at single SSR locus can be objectively visualised and defined. The methods are robust and the results more easily compared between laboratories than other methods. Numerous SSR loci have been generated over the years (e.g. Lees et al., 2006, Knapova et al., 2002). A standardised set of 12 loci were described in 2013 that have been developed as a multiplex PCR assay in which all 12 loci can be amplified in a single reaction and is applicable for pure pathogen DNA or mixed host and pathogen DNA on FTA cards (Li et al., 2013). This 12-plex assay forms the basis of many national (Dey et al., 2018, Njoroge et al., 2019, Li et al., 2012) and international (Martin et al, 2019) comparisons but no standard laboratory protocols have been available until now. In this document the working protocols from the Li et al. (2013) assay used for > 8000 isolates genotyped in the EuroBlight monitoring work and other projects at The James Hutton Institute are described.

General points

1. The protocols describe the use of the 12-plex assay with the alleles sized on an Applied Biosystems 3730 capillary sequencer with a 5-dye system than relies on the FAM, VIC, NED, and PET labelled oligonucleotide primers and a LIZ-based size standard. VIC, NED and PET are only supplied by Applied Biosystems (now Thermo-Fisher in UK). Other types of fluorescent dyes are available but be aware this will affect the allele sizing and may therefore require additional validation steps.
2. The bond between oligonucleotide primers and the fluorescent dye tag is prone to damage by exposure to light and freeze-thaw cycles. Such degradation leads to a weakening of the fluorescent labelling and thus peak heights weaken over time and will yield poor results. Exposure to light MUST be minimised from the outset when diluting and making up aliquots of all labelled primers. On the lab bench, always keep working solutions and completed reactions in covered ice boxes and reduce light exposure to the minimum. We advise working with small aliquots that are

sufficient for 200 reactions (2 plates) only as this minimises freeze-thaw cycles and light exposure. Length variation in the primer batch will lead to less accurate peak size calling so we recommend HPLC purification (i.e. sizing) of the primers. Some primers include the addition of short 5' primer modification called PIGtailing (Brownstein et al., 1996) to facilitate accurate peak calling.

3. Difference in the PCR amplification efficiency of each locus leads to marked variation in the final peak heights. The primer dilutions presented here have been optimised to standardise the peak heights of the alleles at each of the 12 loci. The dilutions represent a guide and may be further optimised in according to differing PCR conditions in your laboratory.
4. We advocate using the QIAGEN Type-It PCR kit. Our preliminary work on the 12-plex with standard *Taq* polymerase resulted in gaps in the 12-plex profiles with 1-2 of the loci failing to amplify. We reduced the reaction size from the manufacturers protocols to make the assay more cost-effective.
5. Use of DNA standards (provided on request from authors) of known pathogen genotypes is critical to calibrate allele calling. See allele scoring guide on EuroBlight website.

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Step by step guide for genotyping 96 samples

Starting material

- Pathogen DNA extracted from freeze-dried or fresh *P. infestans* mycelium using the EtOH protocol (Wang et. al., 1993). We use a concentration of ca. 20 ng per µl. Or:
- A 2mm disk from interface of green leaf and late blight lesion from a lesion pressed onto an FTA card. Washed and dried according to the manufacturers protocol.
- Small aliquots (20µl) of all forward and reverse primers diluted to 100 µM except Pi04 primers diluted to 4.7 µM

Conduct All steps on ice and minimise exposure to light

1. Prepare primer mix and PCR master mix

Prepare Primer mix 1

Comprising 1.25 µl each of forward and reverse primers (100 µM Stocks) of Pi02(SSR3), SSR11, Pi70.
2.0 µl each of forward and reverse primers (100 µM Stocks) of G11
1.5 µl each of forward and reverse primers (100 µM Stocks) of SSR4, Pi63
1.0 µl each of forward and reverse primers (100 µM Stocks) of SSR6, SSR2
1.5 µl of HPLC water
Total volume of 23 µl.
NB 10 µl of this primer mix is used immediately in the master mix below and the rest is frozen ready for the next plate.

Prepare Reaction Master Mix

	X1 (µl)	X100 (I.E. Full PCR plate)
Water	4.81	481
2x Qiagen Type-IT Multiplex PCR Mix	6.25	625
Primer Mix 1	0.1	10
Pi4B Fwd Primer (100 µM stock)	0.03	3
Pi4B Rev Primer (100 µM stock)	0.03	3
SSR8 Fwd Primer (100 µM stock)	0.04	4
SSR8 Rev Primer (100 µM stock)	0.04	4
D13 Fwd Primer (100 µM stock)	0.02	2
D13 Rev Primer (100 µM Stock)	0.02	2
Pi04 Fwd Primer (4.7 µM Stock)	0.08	8
Pi04 Rev Primer (4.7 µM Stock)	0.08	8
Total Volume	11.5 µl	1150 µl

2. Dispense master mix into samples

Add 11.5 µl of master mix to 1 µl of pathogen DNA or add to the well containing the washed and dried FTA card disk.

3. PCR

PCR according to the below protocol. If using FTA cards as a template increase the cycle numbers to 33.

PCR Programme

95°	5 min	
95°	30 sec	28 Cycles
58°	90 sec	
72°	20 sec	
60°	30 min	

4. Prepare plate for sequencing service (capillary allele sizing)

Dilute PCR product 1:20 prior to set up of plate for sequencing service. If really weak PCR template, don't use the full dilution at this point, can even be neat in cases where the DNA concentration is extremely low.

Prepare Liz size standard/ HiDi formamide mix. Add 6 µl of Applied Biosystems Liz500 size standard to 1 ml of pre-prepared HiDi Formamide. (1 ml aliquots of HiDi prepared and frozen for use from the liquid stock supplied to slow breaking down of the chemical).

Dispense 10.2 µl of HiDi/Liz mix into each well of a fresh 96-well PCR plate (Abgene AB0600). Add 0.6 µl of diluted PCR product of each sample to each well. Seal and send to sequencing facility. If using a 48 capillary ABI 3730 machine and you have half a plate or fewer samples, load either even or odd numbered columns of the plate only. Pure water should be used for empty wells in partly used columns (i.e. blanks).

Complete naming of samples on template data entry form for sequencing service layout. Do not use default labelling of samples 1-96 (see SSR help notes)

Reagent list

- Type-it Microsatellite PCR Kit (2000) (Qiagen) — Cat No./ID: 206246
- Applied Biosystems Hi-Di™ Formamide (Thermo-Fisher) — Cat No./ID: 4401457
- Applied Biosystems™ GeneScan™ 500 LIZ™ dye Size Standard (Thermo-Fisher) — Cat No./ID: 4322682
- Whatman® FTA® card classic card (Merck/Sigma Aldrich) — Cat No./ID: WHAWB120205
- Whatman® FTA® purification reagent (Merck/Sigma Aldrich) — Cat No./ID: WHAWB120204
- PCR Plate, 96-well, non-skirted (Thermo-Fisher) — Cat No./ID: AB0600
- Whatman™ WB100007 Harris Micro-Punch with 2mm tip — Cat No./ID: Z755222

Appendix 1

Definitive primer list for 12-Plex assay decided on between David Cooke (JHI, Dundee, UK) and Theo van der Lee (PRI, Wageningen, The Netherlands) August 2011. We order labelled primers from Thermo Fisher via their Custom oligo – Fluorescent labelled primers service at the 80K pmol delivery scale using their HPLC purification method. NB Pi02 and SSR3 are the same locus described by Lees et al and Li et al, respectively.

Pi02(SSR3)	
Pi02_Fwd_NED	NED-ACTTGCAGAACTACCGCCC
Pi02_Rev_PT	GTTTGACCACTTTCCTCGGTTC
SSR11	
SSR11_Fwd_NED:	NED-TTAAGCCACGACATGAGCTG
SSR11_Rev_PT:	GTTTAGACAATTGTTTTGTGGTCGC
SSR4	
SSR4_Fwd_FAM	FAM-TCTTGTTCGAGTATGCGACG
SSR4_Rev_PT	GTTTCACTTCGGGAGAAAGGCTTC
SSR6	
SSR6FwdPT	GTTTTGGTGGGGCTGAAGTTTT
SSR6_Rev_VIC	VIC - TCGCCACAAGATTTATTCCG
Pi63	
Pi63Fwd_VIC	VIC - ATGACGAAGATGAAAGTGAGG
Pi63Rev_long	CGTATTTTCCTGTTTATCTAACACC
SSR2	
SSR2_For_PET	PET-CGACTTCTACATCAACCGGC
SSR2_Rev_PT	GTTTGCTTGGACTGCGTCTTTAGC
PiG11	
PiG11_Fwd_NED	NED-TGCTATTTATCAAGCGTGGG
PiG11_Rev_PT	GTTTCAATCTGCAGCCGTAAGA
Pi70	
Pi70Fwd_VIC	VIC - ATGAAAATACGTCAATGCTCG
Pi70Rev	CGTTGGATATTTCTATTTCTTCG
Pi4B	
Pi4B_Fwd	PET - AAAATAAAGCCTTTGGTTCA
Pi4B_Rev	GCAAGCGAGGTTTGTAGATT
SSR8	
SSR8_Fwd_FAM	FAM-AATCTGATCGCAACTGAGGG
SSR8_Rev_PT	GTTTACAAGATACACACGTCGCTCC
D13	
D13_Fwd	FAM-TGCCCCCTGCTCACTC
D13Rev_long	GCTCGAATTCATTTTACAGACTTG
Pi04	
Pi04_Fwd_VIC	VIC -AGCGGCTTACCGATGG
Pi04_Rev_PT	GTTTCAGCGGCTGTTTCGAC

Appendix 2
Final Primer Concentrations in Genotyping PCR

Primer	Stock Conc µM	Into Primer Mix	µl added to PM	Conc in PM	Conc in final Reaction Mix
Pi02F	100	1	1.25	5.43	0.043
Pi02R	100	1	1.25	5.43	0.043
SSR11F	100	1	1.25	5.43	0.043
SSR11R	100	1	1.25	5.43	0.043
SSR4F	100	1	1.5	6.52	0.052
SSR4R	100	1	1.5	6.52	0.052
SSR6F	100	1	1	4.35	0.035
SSR6R	100	1	1	4.35	0.035
Pi63F	100	1	1.5	6.52	0.052
Pi63R	100	1	1.5	6.52	0.052
SSR2F	100	1	1	4.35	0.035
SSR2R	100	1	1	4.35	0.035
PiG11F	100	1	2	8.70	0.070
PiG11R	100	1	2	8.70	0.070
Pi70F	100	1	1.25	5.43	0.043
Pi70R	100	1	1.25	5.43	0.043
Pi4BF	100	main	0.03		0.24
Pi4BR	100	main	0.03		0.24
SSR8F	100	main	0.04		0.32
SSR8R	100	main	0.04		0.32
D13F	100	main	0.02		0.16
D13R	100	main	0.02		0.16
Pi04F	4.7	main	0.08		0.03
Pi04R	4.7	main	0.08		0.03

Appendix 3

Expected coverage of peak sizes.