Journal of Microbiological Methods 92 (2013) 316–322

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Efficient multiplex simple sequence repeat genotyping of the oomycete plant pathogen *Phytophthora infestans*

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ARTICLE INFO

Article history:
Received 5 September 2012
Received in revised form 29 November 2012
Accepted 29 November 2012
Available online 10 January 2013

Keywords:
Phytophthora infestans
SSR
Multiplex PCR
Population
Genotyping

ABSTRACT

Genotyping is fundamental to population analysis. To accommodate fast, accurate and cost-effective genotyping, a one-step multiplex PCR method employing twelve simple sequence repeat (SSR) markers was developed for high-throughput screening of *Phytophthora infestans* populations worldwide. The SSR markers reported for this species were evaluated and the twelve most informative and easily scored were selected. To accomplish a single step genotyping procedure, we optimized primers, fluorescent labels and PCR conditions to genotype using a capillary electrophoresis system with four fluorescent labels (FAM, NED, PET and VIC) and a labeled LIZ standard for sizing of the SSR fragments. The results obtained using commercially available multiplex PCR kits on a set of reference isolates were in agreement with that obtained using primer pairs in simplex reactions. In testing on many thousands of isolates, we have found the markers appropriate for resolving distinct multilocus genotypes (MLGs) of isolates of European and wider populations. Here we demonstrate the utility of the assay on a set of 19 reference isolates plus 77 others sampled from The Netherlands and Great Britain. In most isolates one to two alleles were observed at each locus but the presence of three alleles at a single locus in some isolates was consistent with increased ploidy. Methods are presented that are appropriate for the analysis of datasets comprising isolates of mixed ploidy levels. We also report on the direct *P. infestans* genotyping from infected field material to collect, store and extract pathogen DNA. A critical step in this multiplex method was the standardization of the protocol between two laboratories in The Netherlands and Great Britain. Reference isolates were exchanged and an allele nomenclature and scoring system agreed. Such co-operation is facilitating the genotyping of international collections of *P. infestans* isolates in wider networks of laboratories and providing the data required to expand an existing international database of pathogen diversity.

1. Introduction

Genotyping pathogen populations poses several challenges, i) in general, large numbers of isolates should be examined and the analysis thus requires a rapid, affordable and robust genotyping method; ii) most analysis is performed at a national level but the pathogens move across national boundaries and standardized methods between laboratories are thus required to integrate datasets; iii) in addition the marker resolution should be sufficiently high to discriminate multilocus genotypes (MLGs) and also identify sub-clonal variation; iv) finally, isolation of the pathogen from infected tissue is time consuming and often a rate-limiting step that could be aided by *in planta* fingerprinting. Co-dominant microsatellites (SSRs) have been used increasingly since the late eighties for applications such as fingerprinting, parentage analyses, genetic mapping or genetic structure analyses (Guichoux et al., 2011; Tenzer et al., 1999). Despite growing competition from new genotyping and sequencing techniques, the use of these versatile and cost-effective markers continues to increase, boosted by successive technical advances. First, next-generation sequencing technologies allow the identification of large numbers of SSR loci at reduced cost in non-model species. Second, methods for multiplexing PCR have improved considerably over the last years, thereby decreasing genotyping costs and increasing throughput. A technical advantage of fluorescence-based SSR genotyping is that several SSRs can be separated simultaneously in a single column by separating the loci by allele size and fluorescent dye labels. In order to reduce the costs, samples may be combined in different ways. In post-PCR multiplexing, also called multi-pooling, the products of individual PCR assays are pooled and run through the capillary together. However, PCR multiplexing is a more efficient approach in which several SSR loci are amplified in a single PCR reaction (Hayden et al., 2008). As a consequence, SSRs are currently the preferred type of marker as they are highly polymorphic, reproducible, neutral, co-dominant, affordable and relatively easy to automate and score. Relationships between

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http://dx.doi.org/10.1016/j.mimet.2012.11.021

individuals may be inferred by examining their allelic differences according to a stepwise mutation model. However, few methods are available that can effectively accommodate variation in ploidy within the population (Bruvo et al., 2004).

Phytophthora infestans is an oomycete plant pathogen that causes the serious late blight disease of potatoes. It was responsible for the crop losses that led to the nineteenth century Irish famine (Woodham-Smith, 1962). *P. infestans* is heterothallic, with A1 and A2 mating types required for mating and the formation of sexual oospores. Sexual recombination generates new genotypes that may prove more difficult to manage due to, for example, the evolution of novel virulence to host resistance genes, fungicide resistance or increased aggressiveness. Prior to the 1980s both mating types were only found together in the Toluca Valley, in Mexico, which may be its center of origin (Grünwald and Flier, 2005). In the early 1980s, the A2 mating type was first found in Europe (Goodwin and Drenth, 1997) and subsequently in many other countries (Fry et al., 1992, 1993). Since this migration, the pathogen population in Europe has undergone a major transition with the pre-1980 population being replaced by new clonal lineages and a move to sexual recombination in some regions such as The Netherlands and Nordic regions (Brurberg et al., 2011; Drenth et al., 1994; Zwankhuizen and Zadoks, 2002). Tracking such population change has involved a range of different methods as reviewed by Cooke and Lees (2004). However, SSR technology is currently the favored approach and has great value in, for example, discriminating MLGs, tracking pathogen spread on local and international scales and examining the presence and significance of sexual recombination. Such studies are providing valuable information on contemporary populations to the benefit of growers, advisors, the agrochemical industry and potato breeders (Cooke et al., 2012a).

Several SSR markers have been used for *P. infestans* population studies (Cooke and Lees, 2004; Knapova and Gisi, 2002; Lees et al., 2006). In 2010 a multiplex SSR set of eight markers was first reported for *P. infestans* (Li et al., 2010). In this study our aim is to combine the most informative and easily scored markers from the previous studies into a one-step multiplex PCR assay. The assay must allow faster, more accurate and cost-effective data acquisition for examining the pathogen on an international scale and ideally be applicable to both pure *P. infestans* DNA as well as being suitable for use on DNA extracted directly from infected plant tissue. We developed the assay in two different laboratories (WUR, Wageningen UR, The Netherlands and JHI, the James Hutton Institute, Great Britain) and standardized the allele binning and nomenclature across all 12 multiplexed loci. This harmonized assay will serve as a single means of scoring *P. infestans* diversity. Importantly, we also explored means of analyzing such data using a sub-sample of European isolates representing clonal and diverse sexual populations that also accommodated the challenges of the variable ploidy levels observed.

2. Materials and methods

2.1. Samples

For this study 96 *P. infestans* isolates were selected to demonstrate the method. Diverse reference isolates are from the previous studies (Lees et al., 2006; Li et al., 2010) and were combined with other samples selected from pathogen surveys as representatives of known clonal lineages and novel and diverse MLGs in both The Netherlands and Great Britain sampled over the period 2001 to 2011 (Table 1 and Supplementary Table S2).

2.2. DNA extraction

Agar plugs of each individual *P. infestans* isolate were taken from the edge of a seven day old actively growing colony in pea agar medium (120 g of frozen peas, 15 g of agar, 1 l of distilled water) and transferred to liquid pea broth (120 g of frozen peas, 1 l of distilled water). After 3–4 days of incubation at 20 °C in the dark, sufficient mycelium was available for DNA extraction. Genomic DNA was isolated from 20 mg of lyophilized mycelium using the DNeasy 96 Plant Kit (QIAGEN, Cat. No. 69181). The procedure followed the detailed manufacturer's instructions and elution was made with 200 µl ultra-pure water. This was also compared to a simple NaOH extraction method (Wang et al., 1993).

Table 1
Reference *P. infestans* isolates used in the study.

Isolate	Source	Origin	Mating type	Year isolated	MLG
T30-4	WUR	–[a]	A1	1992	misc
IPO428-2	WUR	Netherlands	A2	1992	misc
80029	WUR	Netherlands	A1	1980	misc
88133	WUR	Netherlands	A2	1988	misc
VK1.4	WUR	Netherlands	A1	1958	US-1
90128	WUR	Netherlands	A2	1990	misc
IPO-complex	WUR	Belgium	A2	1982	misc
VK98014	WUR	Netherlands	A1	1998	misc
US467 (C6)	W. E. Fry, Cornell Univ.	USA	A2	1998	US-8
EC1 (C7)	G. Forbes, CIP	Ecuador	A1	1998	EC-1
AR4 (C8)	Unknown	Argentina	A2	1997	n/a
96.17.5.3 (C1)	JHI	Scotland	A1	1996	EU-4
95.16.3.1 (C9)	JHI	Scotland	A1	1995	EU-8
95.17.3.2 (C2)	JHI	Scotland	A1	1995	EU-8
97.38.2.2 (C3)	JHI	Scotland	A1	1997	EU-8
96.9.5.1 (C4)	JHI	Scotland	A1	1996	EU-5
96.13.1.3 (C5)	JHI	Scotland	A1	1996	EU-5
97.28.1.2 (C10)	JHI	Scotland	A2	1997	misc
06_3928A	JHI	England	A2	2006	EU-13

[a] This isolate is not a field isolate but derived from a cross between isolates 80029 × 88133.

2.3. In planta genotyping

Two methods were used for *in planta* genotyping, automated DNA isolation (tested in WUR) and FTA cards (in JHI). The automated isolation was used to directly purify DNA from infected leaves using the sbeadex mini plant kit (Agowa, Cat. Nos. 41601 and 41610) and KingFisher 96 instrument (Thermo Scientific). The leaf samples were harvested by punching a section of a fresh foliar blight lesion with the upper part of a blue 1 ml pipette tip. The instruments were used to complete the DNA extraction procedure in approximately 30 min. The other *in planta* method, performed at The James Hutton Institute was to firstly press sap from the margins of actively spreading foliar blight lesions onto FTA Classic cards (Whatman, WB120205) using a pair of pliers. After air-drying, the cards may be used immediately or stored at room temperature for several years. Small disks were cut from the card using a 2 mm Harris Micro Punch (Whatman, WB100007) and processed according to the manufacturer's protocols in FTA purification reagent (Whatman, WB120204) before being placed directly into the PCR mix for multiplex SSR analysis (see below).

2.4. SSR primer design

The twelve SSRs used in this study were selected from the 20 previously published sets according to their map position, ease of scoring and allelic diversity (Knapova and Gisi, 2002; Lees et al., 2006; Li et al., 2010; van der Lee et al., 2004). If required, the primers were redesigned to adjust the PCR product size for the multiplex assay (Table 2) and each locus was assigned one of the four different fluorescent labels (6FAM; NED; VIC; PET, Applied Biosystems) in such a manner that no two markers with the same fluorescent dye had overlapping allele size ranges. Addition of 5′ PIG tail "GTTT" to reverse primers has been reported to reduce the stutter peaks and assist the

Table 2
The SSR primers used in the study and the final concentration of each primer in multiplex PCR reaction.

SSR locus	Dye	Product size range (bp)	Primer sequence	Final con. (μM)	PIC	Rare allele[a]
PiG11[b]	NED	130–180	FwdNED-TGCTATTTATCAAGCGTGGG	0.05	0.78	8/13
			Rev-GTTTCAATCTGCAGCCGTAAGA	0.05		
Pi02/PinfSSR3[c,d]	NED	255–275	FwdNED-ACTTGCAGAACTACCGCCC	0.05	0.55	2/4
			Rev-GTTTGACCACTTTCCTCGGTTC	0.05		
PinfSSR11[e]	NED	325–360	FwdNED-TTAAGCCACGACATGAGCTG	0.05	0.33	1/4
			Rev-GTTTAGACAATTGTTTTGTGGTCGC	0.05		
D13[c]	FAM	100–185	FwdFAM-TGCCCCCTGCTCACTC	0.16	0.66	13/16
			Rev-GCTCGAATTCATTTTACAGACTTG	0.05		
PinfSSR8[e]	FAM	250–275	FwdFAM-AATCTGATCGCAACTGAGGG	0.3	0.50	2/4
			Rev-GTTTACAAGATACACACGTCGCTCC	0.3		
PinfSSR4[e]	FAM	280–305	FwdFAM-TCTTGTTCGAGTATGCGACG	0.05	0.61	3/7
			Rev-GTTTCACTTCGGGAGAAAGGCTTC	0.05		
Pi04[c]	VIC	160–175	FwdVIC-AGCGGCTTACCGATGG	0.05	0.57	1/4
			Rev-GTTTCAGCGGCTGTTTCGAC	0.05		
Pi70[c]	VIC	185–205	FwdVIC-ATGAAAATACGTCAATGCTCG	0.05	0.19	1/3
			Rev-CGTTGGATATTTCTATTTCTTCG	0.05		
PinfSSR6[e]	VIC	230–250	Fwd-GTTTTGGTGGGGCTGAAGTTTT	0.05	0.44	0/3
			RevVIC-TCGCCACAAGATTTATTCCG	0.05		
Pi63[c]	VIC	265–280	FwdVIC-ATGACGAAGATGAAAGTGAGG	0.05	0.58	0/3
			Rev-CGTATTTTCCTGTTTATCTAACACC	0.05		
PinfSSR2[e]	PET	165–180	FwdPET-CGACTTCTACATCAACCGGC	0.05	0.35	1/3
			Rev-GTTTGCTTGGACTGCGTCTTTAGC	0.05		
Pi4B[b]	PET	200–295	FwdPET-AAAATAAAGCCTTTGGTTCA	0.3	0.62	2/5
			Rev-GCAAGCGAGGTTTGTAGATT	0.3		

The equivalent allele sizes are reported in www.euroblight.org. Note Pi02 (Lees et al., 2006) and PinfSSR3 (Li et al., 2010) are the same locus. The primer concentrations are for guidance only and may need to be adjusted for other PCR and electrophoresis conditions in other laboratories.
[a] The ratio of the rare alleles and observed alleles and the frequency of the rare alleles are less than 10%.
[b] Primers previously reported by Knapova and Gisi (2002).
[c] Primers previously reported by Lees et al. (2006).
[d] Primers for Pi02 locus modified to amplify a larger fragment than previously published (Lees et al., 2006).
[e] Primers previously reported by Li et al. (2010).

scoring of length polymorphisms (Brownstein et al., 1996). In the previous study (Li et al., 2010), it was confirmed that the reverse primer of markers PinfSSR2, Pi02/PinfSSR3, PinfSSR4, PinfSSR6, PinfSSR8, and PinfSSR11 offered a benefit. In this study, the benefit of adding a PIG tail to reverse primers of another six SSRs (G11, D13, Pi04, Pi70, Pi63, Pi4B) was tested.

2.5. Primer optimization

The optimal concentration of locus-specific primer required to amplify the target sequence was determined empirically. Initially 0.4, 0.2, 0.1 and 0.05 μM of locus-specific primer was tested. PCR products were separated on 3% agarose gels. The optimal primer concentration was determined by visual inspection as the strong amplification of a PCR fragment of the expected size. In instances where it was desirable to improve PCR specificity and yield, additional locus-specific primer concentrations were tested.

2.6. Multiplex PCR reaction

The amplification of twelve SSRs by uniplex and multiplex PCR was performed under identical reaction conditions. Uniplex PCR was performed in a volume of 20 μl, 10 ng template DNA, 200 μM of each dNTP, 0.08 U Taq DNA polymerase (Roche Diagnostics, Leiden, The Netherlands), 50 mM KCl, 1.5 mM $MgCl_2$, and a range of concentrations (Table 2) of the locus-specific primers. For multiplex PCR, locus-specific primers for several markers were added to each reaction at the optimal concentration determined in uniplex assays. The QIAGEN multiplex PCR kit (QIAGEN, Cat. No. 206145) was used at WUR and the QIAGEN Type-it Microsatellite PCR Kit (QIAGEN, Cat. No. 206243) was used at JHI. Amplification reactions were as described by the manufacturer. Amplifications using the QIAGEN multiplex PCR kit were run in a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA), with an initial denaturation at 95 °C for 15 min, followed by 30 cycles of 95 °C for 20 s, 58 °C for 90 s, and 72 °C for 60 s, and a final extension at 72 °C for 20 min. At JHI using the QIAGEN Type-it Microsatellite PCR Kit the manufacturer's protocols were followed; PCR conditions 95 °C for 5 min followed by 28 cycles (33 cycles for FTA cards) of 95 °C for 30 s, 58 °C for 90 s, and 72 °C for 20 s, and a final extension at 60 °C for 30 min. Several different PCR machines have been tested. At JHI the manufacturer's recommended reaction sizes were successfully reduced to 12.5 μl.

The commercial multiplex PCR kits were key to the success of this multiplex assay. Using standard PCR kits one or two of the twelve loci failed to amplify and the variation in PCR efficiency, and thus peak heights, was greater. Careful design of the PCR product sizes and dyes used, optimization of the primer concentrations and the use of the 5′ PIG tail "GTTT" were also important.

2.7. Error protection

To avoid discrepancies among studies and across time in different laboratories, reference (or control) isolates should be included in each study. In this study DNA of a reference set of eight Dutch isolates (Table 1) was included in each batch of 96 samples to calibrate and account for differences such as polymer batch, buffer concentration, array quality, ambient laboratory temperature, fluorescent label and variation in internal size standards that may affect the reproducibility of denaturing capillary electrophoresis. The reference samples of known SSR marker genotype were amplified alongside the test samples using the same mastermix under the same PCR conditions. Reference isolates and their DNA templates are maintained in the laboratory and should be available for sharing with other groups to standardize allele calling between laboratories.

2.8. SSR scoring and quality control

Electrophoresis and visualization of SSRs were performed on a Geldoc system of Herolab, type RH-5 and ABI3730 DNA analyzer

(Applied Biosystems). Five μl of PCR products was mixed with 1/6 volume of gel loading buffer (Orange G loading buffer) and separated on 3% agarose gel. For ABI3730 analysis at WUR, the PCR products were diluted 1000 times and 1 μl of diluted SSR product was added to 9 μl of deionized formamide (Hi-Di Formamide Part no. 4311320, Applied Biosystems) containing 0.045 μl of GeneScan-500LIZ standard (Part no. 4322682, Applied Biosystems). At JHI the PCR product was diluted 100 times and 0.6 of the diluted product added to a HiDi and GeneScan-500LIZ mix as described above. Samples were run on an automated ABI 3730 capillary sequencer according to the manufacturer's instructions. SSR allele sizing was performed and scored using GeneMapper v3.7 software (Applied Biosystems).

Within the GeneMapper software, kits, panels and binsets were generated defining the markers and their known allele bins (Supplementary Table S1). The required text files for GeneMapper used in this study as well as images of scored peaks and the MLGs of reference isolates are available on the Euroblight website (www.euroblight.net). Once loaded into GeneMapper, the panels and binsets are applied and peak sizes calculated according to the size standard before the manual curation of the reference samples according to their previously assigned allele (i.e., bin) name. If possible the primer and dye combinations should remain fixed as differences between expected and observed reference alleles due to dye shift have been reported (Sutton et al., 2011). Any slight variation in the observed reference allele sizes compared to the expected sizes (within the original binset file) from one laboratory to another was accounted for by adjustment of the bin centers, keeping the allele names the same. The resultant allele call table was exported from GeneMapper for further genetic analysis.

2.9. Data analysis

The Polymorphism Information Content (PIC) value is a commonly used measure of the amount of information one can recover from a genetic marker. The PIC values for each SSR were estimated from the number and frequency of alleles per locus using the following formula (Anderson et al., 1993; Botstein et al., 1980):

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} Pi^2,$$

in which k is the total number of alleles detected for one SSR marker and Pi is the frequency of the ith allele.

Specialized genetic distances, termed Bruvo distances (Bruvo et al., 2004), and principal coordinate analysis (PCO) were calculated using POLYSAT (Clark and Jasieniuk, 2011) implemented in R. The distance matrix was exported from POLYSAT for further analysis to either generate a minimum spanning tree in MINSPNET (Excoffier and Smouse, 1994) or a neighbor joining tree in NEIGHBOR in PHYLIP (Anderson et al., 1993; Felsenstein, 1989). Minimum spanning trees were drawn as part of GraphViz (http://www.graphviz.org) and neighbor joining trees viewed and exported using FigTree 3.1 (http://tree.bio.ed.ac.uk/). The first two principal coordinates were exported from R and plotted in GenStat for Windows (14th Edition).

3. Results

3.1. Primer optimization

To optimize scoring of the length polymorphism the 5′ PIG tail "GTTT" was added to the unlabeled primer sequences. For PinfSSR2, PinfSSR3, PinfSSR4, PinfSSR6, PinfSSR8, PinfSSR11, it has been previously confirmed that this can reduce stutter peaks (Cooke and Lees, 2004; Lees et al., 2006; Li et al., 2010). In this study, PIG tails were tested on primers for markers G11, D13, Pi04, Pi70, Pi63 and Pi4B. In the case of G11 and Pi04 the 5′ PIG tail improved the quality of scoring as the "stutter" of the main SSR peaks is reduced. For the other SSR loci, no improvement was noted and the modified primers were not used further.

3.2. The development of multiplex PCR with 12 SSRs

The reaction conditions for the PCR assay were optimized to ensure that all the target gene sequences were satisfactorily amplified. To achieve strong amplification of a PCR fragment(s) of the expected size, the optimal concentration of each locus-specific primer pair was determined. Initial tests of a standard concentration (0.1 μM) of each set of primers resulted in uneven peak intensities or absence of amplification of some markers. To overcome this, the proportions of the various primers in the reaction mixture were adjusted with the final concentration varying from 0.05 μM to 0.3 μM (Table 2). All PCR primers were prepared as 10 mM stock solutions which were frozen at −20 °C in 20 μl aliquots to minimize thawing and freezing of the reagents. All 12 SSR loci in the multiplex PCR were successfully amplified as assessed by ABI3730 fluorescence intensities that fell within a peak height range optimal for semi-automated allele sizing, i.e. 500–5000 relative fluorescence units with minimal stutter peaks.

3.3. Robustness of multiplex PCR (genotyping error and null alleles)

To assess the robustness of multiplex PCR to variation in the concentration and quality of DNA samples, two sets of comparisons were completed. Firstly, the effect of DNA concentration and quality were determined by comparing the different DNA extraction methods (i.e. DNeasy kit and NaOH extraction from dried pathogen mycelia, FTA card and direct automated isolation from infected leaves). We observed differences in peak height depending on the concentration and quality of the extracted DNA but peak size was not affected. This indicates that the 12-plex method is robust and insensitive to genotyping error due to variable DNA quality or quantity (data not shown). A step by step protocol is published online (see www.euroblight.net).

Secondly, the multiplex PCR has been applied to over 5000 isolates of global origin in the WUR (NL) and JHI (UK) laboratories. The error rates were negligible among reference isolates that were run in every batch of samples (WUR, NL). Furthermore, many hundreds of isolates of some dominant clonal lineages have been genotyped with this system and the allele sizes and peak height ratios recorded were identical at a wide range of DNA concentrations in extractions using a range of methods. The standard reference allele names and peak sizes reported to date are presented (Supplementary Table S1) and will be continually updated on the Euroblight website (see protocols section of www.euroblight.net). Null alleles were called in cases where amplification of only a single locus in the 12-plex assay failed repeatedly, i.e. a technical failure can be excluded. To date, null alleles have only been recorded at a low frequency in the markers D13, G11 and PinfSSR8. Reference samples have been run using both the QIAGEN multiplex kits mentioned above with identical results. Similarly, we have found that the same isolates run on several different PCR machines generated identical fingerprints.

3.4. Case study: the analysis of SSR data from 96 isolates

The reference isolates and those selected from NL and UK populations were fingerprinted using the 12-plex assay and the findings reported as a case study to illustrate the potential of the method. It was not the intention of this study to explore the specific details of the populations in question. The samples were chosen as representative of clonal lineages (Cooke et al., 2012a; Fry et al., 2009), some of which are known to be present in both The Netherlands and Great Britain and a mixed group of diverse isolates whose fingerprints (Supplementary Table S2) are consistent with them originating from a population undergoing sexual recombination (Brurberg et al.,

2011). The latter set was defined as 'miscellaneous' (hereafter 'misc'). The PIC levels of the markers, as determined in this dataset, ranged from 0.19 (Pi70) to 0.78 (G11) and 80 distinct fingerprint patterns (MLGs) were discriminated among the 96 isolates. Three peaks were recorded at one or more loci in 26 of the 96 isolates which suggests that different ploidy levels were present in the sampled population. This is consistent with previous studies of altered levels of ploidy or trisomy of *P. infestans* (Tooley and Therrien, 1991; van der Lee et al., 2004). Such a mixture of isolates of differing levels of ploidy complicates the analysis of the resultant SSR data (Bruvo et al., 2004; Clark and Jasieniuk, 2011). The R package POLYSAT infers the ploidy from the number of alleles at a locus and calculates a genetic distance matrix and principal coordinate analysis of populations of mixed ploidy levels. A plot of the first two principal coordinates exported from POLYSAT (Fig. 1) provides an overview of the variance of the dataset and indicates a broad scatter of data points among isolates defined as 'misc' with clusters of isolates representing the clonal types. In particular, minor variation was observed among a cluster of 15 isolates defined as the EU_13 MLG (equivalent to 'Blue_13' or 13_A2 (Cooke et al., 2012b; Fry et al., 2009). The variation in such cases was predominantly at highly polymorphic loci with high PIC values (e.g. D13, G11 and PinfSSR4). The dendrogram indicating the genetic relatedness of the isolates (Fig. 2) similarly indicates considerable diversity among the collection with an overall 'star' or 'bush-like' shape indicating there is no clear sub-structuring among this sample of 96 isolates. As in the case of the PCO, clusters of closely related isolates representing minor variants of clonal types were evident. An additional analysis using minimum spanning trees indicated a broadly similar pattern (data not shown).

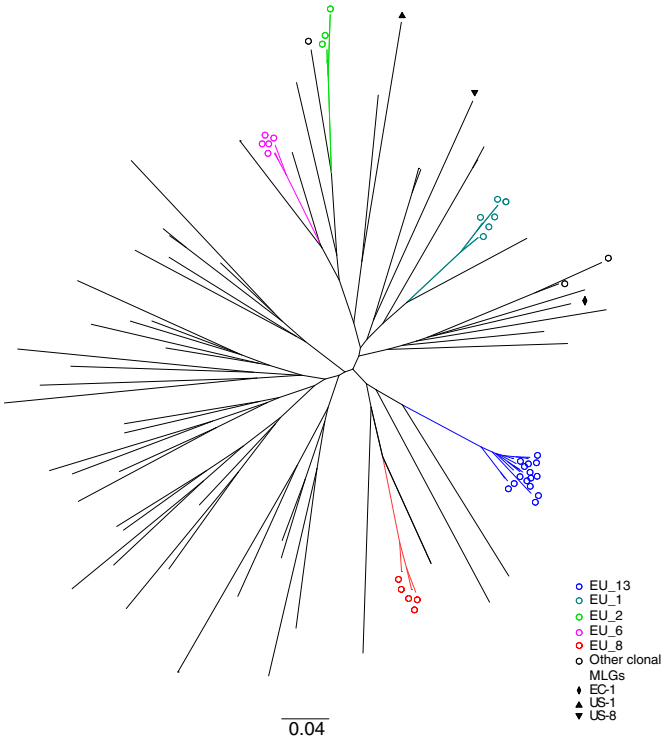

Fig. 2. The NJ dendrogram of the genetic diversity of 96 isolates. The isolates of some clonal lineages are marked with symbols and the branches with no labels represent 'misc' isolates.

3.5. Determination and naming of clonal lineages

On the basis of a range of marker systems including SSR, mitochondrial haplotype and mating type, some *P. infestans* isolates can be grouped into distinct clonal lineages. These clonal lineages of *P. infestans* were determined in dendrograms with significant bootstrap support based on multilocus SSR data supplemented with that of mitochondrial markers and mating types (data not shown). In accordance with other proposals (Grünwald et al., 2008), we suggest that lineages are named with a two letter identifier for the region in which they were first found (e.g., EU = Europe, CN = China) followed by a number indicating the order in which they were described. In such a system lineage EU_13 would be the name for what was called 13_A2, a lineage of mating type A2 and mtDNA haplotype Ia that was first reported in Europe (Cooke et al., 2012a). We do not propose standardizing nomenclature for different genotypes in this study but the standard nomenclature and alleles of genotypes will be presented in detail on the Euroblight website.

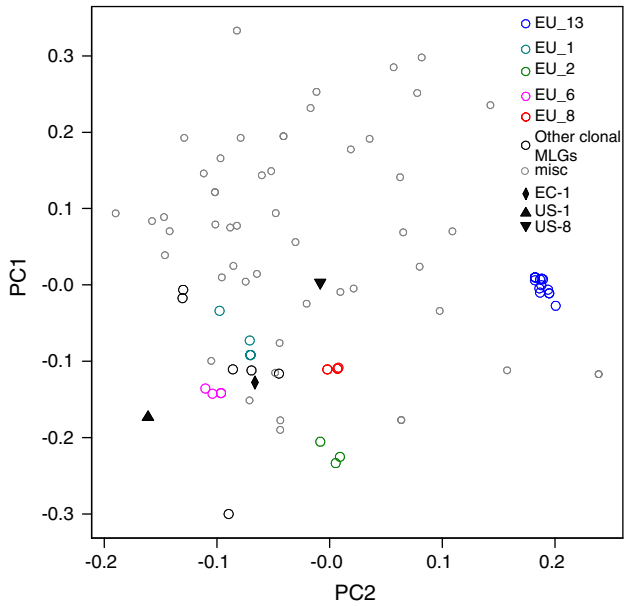

Fig. 1. A plot of the first two principal coordinates representing 96 selected isolates which were characterized using 12 SSR markers and examined in POLYSAT.

3.6. Fast and high-throughput within-season in planta fingerprinting

Conventional typing of pathogens from plant samples takes a minimum of seven days with the establishment of pure pathogen cultures from which DNA is extracted generally being the rate limiting step. An alternative that can provide more rapid feedback to growers within a season is to examine the pathogen DNA directly from lesions using either automated or FTA-based extraction methods. In this study we have comprehensively tested over 1000 lesions using these two methods and find that both are very effective with as many as 90% of lesions successfully fingerprinted. To account for the lower levels of DNA released from the 2 mm diameter disk cut from the FTA card, additional cycles of PCR are added according to the QIAGEN kit instructions. Both provide great potential for a rapid, easy, high-throughput and inexpensive detection pipeline for tracking pathogen populations within a field season and are ideal for 'mark and recapture' type experiments to study in-field pathogen fitness and competition (Cooke et al., 2012a). FTA cards also allow the convenient typing of lesions from remote sites without access to laboratories and materials for pathogen isolation.

4. Discussion

Fluorescence-based SSR detection and allele sizing on an automated DNA fragment analyzer is one of the most rapid and accurate

methods for SSR genotyping. The assay described in this study is a one-step multiplex PCR amplification to facilitate highly parallel, fluorescence-based genotyping of 12 SSR loci in isolates of *P. infestans*. The reproducibility and resolution of the assay have been demonstrated on a diverse collection of isolates from different countries by the two laboratories. Microsatellite fingerprint data for over 5700 isolates of *P. infestans* from Europe have been submitted to the Eucablight database (www.eucablight.org) to date. We propose to expand this database within the Euroblight (www.euroblight.net) platform (Cooke et al., 2012b) and encourage users to upload data generated using the 12-plex assay described in this paper. Only data with predefined SSR allele names may be uploaded. For a new allele to be authenticated, partners should ideally distribute the DNA sample, raw data sample file (e.g. ABI.fsa file) or an image of the new allelic peaks, to a reference laboratory such as WUR or JHI.

Within some regions of Europe, widely distributed clonal lineages have been identified on the basis of fixed lineage-specific combinations of alleles defining each MLG (Cooke et al., 2012a). However, as seen in this study, divergence at the more rapidly evolving SSR loci among isolates within a given MLG is apparent; the 15 isolates of EU_13, for example, vary at loci G11, D13 and PinfSSR4 (Fig. 2; Table S2). Such differences within clonal lineages of other *Phytophthora* species have been reported (Goss et al., 2009). The markers in this 12-plex assay thus provide a useful mix of loci with a lower mutation rate (those in open reading frames) and those that evolve more rapidly (those in non-coding regions and with longer repeat units).

In the case of diploid taxa, co-dominant markers such as SSRs yield datasets that are appropriate for detailed population genetic analysis. In this and other studies using SSR markers on *P. infestans* (Brurberg et al., 2011) three alleles have been noted in some isolate/marker combinations (Table S2). In cases where a third allele was observed (26 of 96 isolates) we also noted that peak height ratios in other heterozygous loci of the same isolate were skewed from the expected 1:1 ratio towards 2:1 (data not shown; Cooke et al., 2012b). Such data is consistent with this sample of 96 isolates comprising a mixture of triploid and diploid isolates. Methods that account for the process of stepwise mutation of alleles to calculate the genetic distances to examine relationships among individuals in such mixed populations became available in 2004 (Bruvo et al., 2004). Clark and Jasieniuk (2011) facilitated the calculation of such 'Bruvo distance' with the publication of POLYSAT, an application in the R environment. In this study we demonstrate the use of POLYSAT to generate appropriate genetic distance measures and a principal coordinate analysis of the sample dataset. The PCO analysis provides an overview of the variance in the data, indicating the broad spread of diverse 'misc' isolates compared to tightly grouped variants of clonal MLGs (Fig. 1). A PCO analysis of SSR data in Nordic regions indicated an even greater diversity and no clustering according to region (Brurberg et al., 2011). The Neighbor Joining tree calculated from the Bruvo distance is a similarly powerful means of displaying clusters of isolates that are variants of clonal lineages. For example, the group of 15 isolates representing variant forms of MLG EU_13 (or 13_A2) is clearly resolved. However, the deeper roots of the tree will not be truly representative of the longer term evolutionary history of these lineages as SSRs are less suited than sequence analysis for this purpose (Guichoux et al., 2011). In populations with large numbers of clones, and variants of those clones, minimum spanning trees (or networks) that present nodes may be more appropriate (Bruvo et al., 2004). In such a system, nodes of different sizes (in proportion to the number of isolates of MLGs) are displayed and the links between these nodes represent the step-wise mutation of the clonal lineage. Such studies have proved suitable for *Phytophthora ramorum* analysis (Goss et al., 2009; Vercauteren et al., 2010) and will be appropriate for largely clonal *P. infestans* populations. In addition, the data may be converted to a binary presence/absence format and treated as dominant markers that can also be used as input for other population analysis applications. A disadvantage of this approach is that relatedness estimates based on the stepwise mutation model are lost in the conversion to a presence/absence matrix that treats the data as dominant markers.

In conclusion, this 12-plex SSR protocol and the associated reference material and analysis tools will enable a more rapid typing of the populations of this destructive pathogen. The resultant data will, for example, enable the source of primary inoculum to be determined and allow the tracking of aggressive lineages with specific traits that influence the effectiveness of current management practices and foster epidemiological studies on a global scale.

Acknowledgments

The authors acknowledge Trudy van den Bosch, Marieke Förch and Geert Kessel in Plant Research International for providing isolates; Marga van Gent-Pelzer and Tineke Veenendaal for work on the assay at WUR, Wageningen; and Naomi Williams and Julie Squires for work on the assay at The James Hutton Institute, Dundee. David Cooke acknowledges funding from the Scottish Government and the Potato Council.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2012.11.021.

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