Identification of the Dominant Genotypes of *Phytophthora infestans* in Canada Using Real-Time PCR with ASO-PCR Assays

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Abstract

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Phytophthora infestans, a pathogenic oomycete that is the causal agent of potato and tomato late blight, has devastating effects worldwide. The genetic composition of *P. infestans* populations in Canada has changed considerably over the last few years, with the appearance of several new genotypes showing different mating types and sensitivity to the fungicide metalaxyl. Genetic markers allowing for a rapid assessment of genotypes from small amounts of biological material would be beneficial for the early detection and control of this pathogen throughout Canada. Mining of the *P. infestans* genome revealed several regions containing single-nucleotide polymorphisms (SNP) within both nuclear genes and flanking sequences of microsatellite loci. Allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) assays were developed from 14 of the 50 SNP found by sequencing. Nine optimized ASO-PCR assays were validated using a blind test comprising *P. infestans* and other *Phytophthora* spp. The assays revealed diagnostic profiles unique to each of the five dominant genotypes present in Canada. The markers developed in this study can be used with environmental samples such as infected leaves, and will contribute to the genomic toolbox available to assess the genetic diversity of *P. infestans* at the intraspecific level. For late blight management, early warning about *P. infestans* genotypes present in potato and tomato fields will help growers select the most appropriate fungicides and application strategies.

Phytophthora infestans (Mont.) de Bary is a pathogenic oomycete with devastating effects globally. It is the causal agent of potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) late blight, diseases for which outbreaks or epidemics have occurred in many countries since the 19th century (Bourke 1964; Fry 2008). This pathogen is notably responsible for the Irish potato famine in 1845, and still has significant effects on potato and tomato production today (Bourke 1964; Fry 2008; Haverkort et al. 2008; Nowicki et al. 2012).

P. infestans is a heterothallic, diploid microorganism that can reproduce both clonally and sexually (Fry 2008). Clonal reproduction is commonly observed in North America, South America, Asia, Africa, and parts of Europe (Fry 2008; Fry et al. 2009). It was believed that sexual reproduction of this organism was mainly restricted to Scandinavia and the Toluca Valley in Mexico (Brurberg et al. 2011; Fry 2008; Fry et al. 2009; Grünwald and Flier 2005; Sjöholm et al. 2013; Yuen and Andersson 2013). However, recent evidence for recombination suggests that sexual reproduction might be contributing to the population structure of this pathogen in other parts of the world, including the United States and Canada (Danies et al. 2014; Kalischuk et al. 2012; Peters et al. 2014).

Many types of tools are available to characterize phenotypic and genetic diversity in *P. infestans* (Cooke and Lees 2004). Among the most widely used are mating type (A1 or A2), sensitivity to the fungicide metalaxyl (Dowley and O'Sullivan 1981), allozymes (Goodwin et al. 1994), restriction fragment length polymorphism (RFLP) fingerprints with the RG57 probe (Goodwin et al. 1992), mitochondrial haplotypes (Carter et al. 1990; Gavino and Fry 2002; Griffith and Shaw 1998), and microsatellites (Knapova and Gisi 2002; Lees et al. 2006; Li et al. 2010, 2013). These techniques are routinely employed to detect new clonal lineages or genotypes, to characterize *P. infestans* populations, to uncover recombination and sexual reproduction, and to document migration events (Cooke and Lees 2004; Fry 2008; Fry et al. 2009). Despite their usefulness, most of these tools share a common disadvantage that complicates the characterization of *P. infestans* diversity. With the exception of microsatellites, the methods all require the culturing of *P. infestans* on appropriate media before testing, which is time consuming and may sometimes be unsuccessful. Although microsatellites have good resolving power and can amplify from small amounts of DNA, technical setbacks such as protocol standardization and scoring calibration have reduced their portability and usage across laboratories (Guichoux et al. 2011).

Single-nucleotide polymorphisms (SNP) are biallelic, codominant genetic markers that result from point mutations producing single base-pair differences among chromosome sequences in a genome (Brumfield et al. 2003). These markers have several advantages such as their abundance in the genome, easiness to score, and potential amenability to different typing methods such as real-time polymerase chain reaction (PCR) assays (Brumfield et al. 2003; Guichoux et al. 2011; Morin et al. 2004). In recent years, real-time PCR assays have shown potential for sensitive and specific detection of *Phytophthora* spp. (Bilodeau et al. 2007b, 2014; Martin et al. 2009, 2012a). Allele-specific oligonucleotide (ASO)-PCR assays designed from SNP (Bottema and Sommer 1993; Green et al. 1991; Newton et al. 1989) and used with real-time PCR technology are very sensitive tests that can be used with samples containing small amounts of mixed DNA, such as environmental samples, making them well suited for rapid genotyping of plant pathogens (Bilodeau et al. 2007a; Gagnon et al. 2014).

Major genetic changes have been observed in populations of *P. infestans* in Canada over the last 30 years. Prior to the mid-1990s, populations of this pathogen were mostly composed of a single genotype (US-1) that is sensitive to metalaxyl and has only the A1 mating type (Goodwin et al. 1994, 1996). During the 1990s, a new genotype (US-8) appeared in Canada. This genotype, with the A2

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*The *e*-Xtra logo stands for "electronic extra" and indicates that six supplementary tables are published online.

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Table 1. Isolates of *Phytophthora infestans* and other *Phytophthora* spp. used for sequencing (Seq), development (Dev), validation (Val), and standard curves (SC) or inoculation experiments with allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) assays[a]

Isolate	Species	Origin	Year	Source[b]	Genotype[c]	Seq	Dev	Val	SC[d]
LRC-1	*Phytophthora infestans*	Canada	2011	AAFC-L	US-8	X	…	X	…
LRC-2	*P. infestans*	Canada	2011	AAFC-L	US-8	X	…	X	…
LRC-3	*P. infestans*	Canada	2011	AAFC-L	US-8	X	…	X	…
LRC-4	*P. infestans*	Canada	2011	AAFC-L	US-11	X	…	…	…
LRC-5	*P. infestans*	Canada	2011	AAFC-L	US-11	X	…	X	…
LRC-6	*P. infestans*	Canada	2011	AAFC-L	US-11	X	…	X	…
LRC-7	*P. infestans*	Canada	2011	AAFC-L	US-23	X	…	…	…
LRC-8	*P. infestans*	Canada	2011	AAFC-L	US-23	X	…	X	…
LRC-9	*P. infestans*	Canada	2011	AAFC-L	US-23	X	…	X	…
DAOM 238142	*P. infestans*	Canada	2009	CCFC	–	X	…	…	…
P1296	*P. infestans*	Ireland	1980	WPC	US-1	X	…	X	…
P6749	*P. infestans*	Mexico	–	WPC	–	X	…	…	…
P8372	*P. infestans*	United States	–	WPC	US-6	X	…	X	…
P9175	*P. infestans*	United States	1995	WPC	US-11	X	…	…	…
P10106	*P. infestans*	United States	1994	WPC	US-7	X	…	X	…
P10119	*P. infestans*	Canada	1996	WPC	US-13	X	…	…	…
Pi-01	*P. infestans*	Canada	2012	AAFC-L	US-23	X	X	X	
Pi-02	*P. infestans*	Canada	2012	AAFC-L	US-23	X	X	…	…
Pi-03	*P. infestans*	Canada	2012	AAFC-L	US-23	X	…	X	X
Pi-04	*P. infestans*	Canada	2012	AAFC-L	US-11	X	X	X	…
Pi-05	*P. infestans*	Canada	2012	AAFC-L	US-11	X	X	X	…
Pi-06	*P. infestans*	Canada	2012	AAFC-L	US-11	X	…	X	X
Pi-07	*P. infestans*	Canada	2012	AAFC-L	US-8	X	X	X	…
Pi-08	*P. infestans*	Canada	2012	AAFC-L	US-8	X	X	X	…
Pi-09	*P. infestans*	Canada	2012	AAFC-L	US-8	X	…	X	X
Pi-10	*P. infestans*	Canada	2012	AAFC-L	US-22	X	X	…	…
Pi-11	*P. infestans*	Canada	2012	AAFC-L	US-22	X	X	…	…
Pi-12	*P. infestans*	Canada	2012	AAFC-L	US-22	X	…	X	X
Pi-13	*P. infestans*	Canada	2012	AAFC-L	US-24	X	X	X	…
Pi-14	*P. infestans*	Canada	2012	AAFC-L	US-24	X	X	X	…
Pi-15	*P. infestans*	Canada	2012	AAFC-L	US-24	X	…	…	X
PiUS23NB	*P. infestans*	Canada	–	AAFC-S	US-23	…	…	X[e]	…
Pi2011-072NB	*P. infestans*	Canada	2011	AAFC-S	US-24	…	…	X[e]	…
US110093	*P. infestans*	United States	2011	Cornell	GDT-06	…	…	X	…
US110074	*P. infestans*	United States	2011	Cornell	GDT-04	…	…	X	…
US110072	*P. infestans*	United States	2011	Cornell	GDT-07	…	…	X	…
US100023	*P. infestans*	Canada	2010	Cornell	GDT-13	…	…	X	…
US110061	*P. infestans*	United States	2011	Cornell	GDT-05	…	…	X	…
US100033	*P. infestans*	Canada	2010	Cornell	GDT-16	…	…	X	…
US100034	*P. infestans*	United States	2010	Cornell	GDT-20	…	…	X	…
US110086	*P. infestans*	United States	2011	Cornell	GDT-03	…	…	X	…
US110085	*P. infestans*	United States	2011	Cornell	GDT-11	…	…	X	…
US100029	*P. infestans*	United States	2010	Cornell	GDT-12	…	…	X	…
US100032	*P. infestans*	Canada	2010	Cornell	GDT-15	…	…	X	…
US110084	*P. infestans*	United States	2011	Cornell	GDT-01	…	…	X	…
US100019	*P. infestans*	United States	2010	Cornell	GDT-19	…	…	X	…
US110064	*P. infestans*	United States	2011	Cornell	GDT-02	…	…	X	…
US110092	*P. infestans*	United States	2011	Cornell	GDT-17	…	…	X	…
US110071	*P. infestans*	United States	2011	Cornell	GDT-08	…	…	X	…
US110082	*P. infestans*	United States	2011	Cornell	GDT-09	…	…	X	
1351798	*P. pseudosyringae*	United States	2005	CDFA	N/A	…	…	X	…
P3009	*P. mirabilis*	Mexico	1987	WPC	N/A	…	…	X	…
BR 255	*P. nicotianae*	–	1977	CCFC	N/A	…	…	X	…
BR 529	*P. megasperma*	United Kingdom	1949	CCFC	N/A	…	…	X	…
BR 675	*P. cactorum*	Canada	1989	CCFC	N/A	…	…	X	…
CBS 101553	*P. ramorum*	Germany	1995	CBS	N/A	…	…	X	…
DAOM 236393	*P. quercina*	Italy	1996	CCFC	N/A	…	…	X	…
BR 518	*P. citricola*	Taiwan	1926	CCFC	N/A	…	…	X	…

[a] Symbol: – indicates missing data (not available).

[b] AAFC-L = Agriculture and Agri-Food Canada, Lethbridge, AB, Canada; CCFC = Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, ON, Canada; WPC = World *Phytophthora* Collection, University of California, Riverside; AAFC-S = Agriculture and Agri-Food Canada, St-Jean-sur-Richelieu, QC, Canada; Cornell = Cornell University, Ithaca, NY; CDFA = California Department of Food and Agriculture, Sacramento; and CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[c] Genotypes based on mating type, allozymes, DNA fingerprints using the RG57 probe, mitochondrial haplotypes, microsatellites, or sequencing of nuclear genes (Danies et al. 2014; Goodwin et al. 1992, 1994; Li et al. 2013; Martin et al. 2012b). N/A = not applicable because not *P. infestans* isolates.

[d] Standard curve of nine optimized ASO-PCR assays.

[e] Used in the inoculation experiments to test the nine optimized ASO-PCR assays with environmental material.

mating type, is highly aggressive on potato and resistant to the fungicide metalaxyl (Goodwin et al. 1996, 1998; Kato et al. 1997). This *P. infestans* genotype dominated in potato fields for nearly 20 years (Fry et al. 2009). Since 2009, increased incidence of late blight in Canada has coincided with the appearance of several new genotypes (US-11, US-22, US-23, and US-24) comprising both mating types and showing different levels of sensitivity to metalaxyl (Kalischuk et al. 2012; Kawchuk et al. 2011; Peters et al. 2014). For example, it was observed in Canada that the US-11 genotype was resistant to metalaxyl while the US-22, US-23, and US-24 genotypes were sensitive to the fungicide (Kalischuk et al. 2012). The increase in frequency of late blight outbreaks, the presence of strains possessing different characteristics, and the co-occurrence of A1 and A2 mating types in some regions poses a challenge for late blight management. A new genotyping tool that can detect the pathogen from very small amounts of DNA and facilitate rapid identification of the dominant genotypes of *P. infestans* in Canada would be very useful for late blight management decisions, such as the choice of fungicide to apply based on knowledge of the genotype fungicide resistance level, and frequency of fungicide applications based on virulence of the identified genotype.

The objectives of this study were to (i) develop ASO-PCR assays for identification of the five dominant genotypes of *P. infestans* present in Canada, (ii) validate the optimized ASO-PCR assays using a blind test comprising *P. infestans* and other *Phytophthora* spp., (iii) validate the optimized ASO-PCR assays using potentially recombinant *P. infestans* isolates from the United States and Canada, and (iv) test the optimized ASO-PCR assays using infected plant material.

Materials and Methods

To achieve these objectives, SNP found in nuclear genes and flanking microsatellite regions that were variable among the five dominant Canadian genotypes of *P. infestans* were used to design primers that were then tested for their utility in ASO-PCR assays.

Sampling. *P. infestans* isolates representing genotypes from Canada and other countries were obtained as listed in Table 1. The clonal lineages or genotypes reported are based on mating type, allozymes (Goodwin et al. 1994), DNA fingerprints using the RG57 probe (Goodwin et al. 1992), mitochondrial haplotypes (Martin et al. 2012b), microsatellite loci (Li et al. 2013), or sequencing of nuclear genes (Danies et al. 2014).

Culturing and DNA extraction were performed by different persons (Table 1) following different protocols. The 2011 and 2012 *P. infestans* isolates from Canada were grown and extracted as described by Kalischuk et al. (2012). The 2010 and 2011 potentially recombinant *P. infestans* isolates from the United States and Canada were grown and extracted as described by Danies et al. (2013). DNA from other *P. infestans* isolates was extracted from frozen mycelia following the Wizard Magnetic DNA Purification System for food (Promega Corp., Madison, WI), with volumes of solutions adapted for mycelial material, and the KingFisher ml instrument (Thermo Fisher Scientific Inc., Waltham, MA). DNA concentration was measured with a Qubit (Life Technologies Inc., Grand Island, NY).

DNA sequencing and ASO primer design. Several genes and flanking microsatellite regions were amplified and sequenced to look for SNP that distinguish one or several of the dominant Canadian *P. infestans* genotypes (US-8, US-11, US-22, US-23, and US-24). Selected loci were PCR amplified using the primers listed in Table 2 and Supplementary Table S1.

For nuclear genes, amplification reactions were carried out in 25-µl volumes with the following final concentrations: 1× buffer, 0.2 mM dNTP, 0.2 µM each primer, 2 mM $MgCl_2$, 0.04 U of Platinum Taq DNA polymerase (Life Technologies Inc.), and approximately 2 ng

Table 2. Nuclear genes and microsatellite flanking regions (MFR) used for sequencing and single-nucleotide polymorphism (SNP) mining in *Phytophthora infestans*

Name[a]	Primer name	Primer sequence	T_a (°C)[b]	Scored bp	Total SNP	Variable SNP[c]	Source	GenBank[d]
Nuclear genes								
ARP2/3	ARP23_For	TAYCCGCCCTACAAGACG	55	864	3	1	Blair et al. (2012)	KM522965–KM522995
	ARP23_Rev	CTTCTGGGTCTTGGACTGGT	…	…	…	…	…	…
PUA	PUA_For	AGGTCAAGTCCTCGCAGCAG	67	583	7	7	Blair et al. (2012)	KM522930–KM522960
	PUA_Rev	AGGTCGTCRCCMAGGAAGTG	…	…	…	…	…	…
Ras	Ras_For	CGTGTCTGCTTCTCCGTTTCG	55	549	6	6	Gómez-Alpizar et al. (2007)	KM523098–KM523128
	Ras_Rev	CCAGGCTTTCGGCAAATTCC	…	…	…	…	…	…
Ras intron 1	RasInt_For	TTGCAGCACAACCCAAGACG	55	288	6	6	Gómez-Alpizar et al. (2007)	KM522996–KM523008
	RasInt_Rev	TGCACGTACTATTCGGGGTTC	…	…	…	…	…	…
TRP1	Trp_For	GCCGCCAAGCAGGTCRT	62	525	3	3	Blair et al. (2012)	KM523017–KM523047
	Trp_Rev	RAYGCTGTTCACCTCSACCA	…	…	…	…	…	…
MFR								
3027R	3027R_fseq	CCCACCGATCACTCCACCT	TD	495	1	1	Abbott et al. (2010)	KM522926–KM522929
	3027R_rev	CGTGGGCAACTACATGACCG	…	…	…	…	…	…
3157F	3157F_for	CGCGATGATGTGGAAGATGG	TD	507	3	3	Abbott et al. (2010)	KM523013–KM523016
	157F_rseq3	TGTAAACCATGCTCTACGATT	…	…	…	…	…	…
3279F	3279F_fseq	TGTGTATATTTATCGAAAGCA	TD	436	5	4	Abbott et al. (2010)	KM523048–KM523066
	3279F_rseq	GCAAAATCGCATTATGTGGT	…	…	…	…	…	…
3332F	3332F_for	ACTGAGACCACCACGCCACC	TD	398	8	7	Abbott et al. (2010)	KM523067–KM523097
	3332F_rseq2	ATCCTTCTCCTCCTTGTCCA	…	…	…	…	…	…
3345F	3345F_for	TGGAGATCGGCGTACCAAGG	TD	360	5	5	Abbott et al. (2010)	KM522961–KM522964
	3345F_rseq	GGATGGGATGCTGTGCTTAG	…	…	…	…	…	…
3350_9F	3350_9F_for	TGCGTTATTGTGCCACCCAC	TD	477	3	3	Abbott et al. (2010)	KM522922–KM522925
	3350_9F_rseq2	GGTCTATTTGCGCTTCTTCT	…	…	…	…	…	…
3361_7R	3361_7R_fseq	GGCCTTTTTAAACGTTTGGA	TD	420	5	5	Abbott et al. (2010)	KM523009–KM523012
	3361_7R_rseq	GCATTTCATTCAAACACAG	…	…	…	…	…	…
Total	…	…	…	…	55	51	…	…

[a] ARP2/3 = actin-related protein 2/3 complex, PUA = conserved hypothetical protein, Ras = Rab 1 family GTPase PiYPT1, and TRP1 = N-(5′-phosphoribosyl) anthranilate isomerase indole-3-glycerol-phosphate synthase.
[b] Annealing temperature. TD = touchdown: annealing temperature from 65 to 53°C (dropping 3°C per three cycles; see Material and Methods).
[c] SNP showing variation among the five dominant Canadian genotypes.
[d] GenBank accession numbers.

of template DNA. PCR conditions were as follows: 2 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at the locus-specific annealing temperature (Table 2), and 1 min at 72°C; and 5 min at 72°C.

For flanking microsatellite regions, amplification reactions were carried out in 10-µl volumes with the following final concentrations: 1× buffer, 0.1 mM dNTP, 0.1 µM each primer, 2 mM $MgCl_2$, 0.05 U of Platinum Taq DNA polymerase (Life Technologies Inc.), and approximately 2 ng of template DNA (Abbott et al. 2010). PCR conditions were 3 min at 95°C; a "touchdown" of 30 s at 95°C, 30 s at 65 to 53°C (dropping 3°C per three cycles), and 90 s at 72°C; 25 cycles of 30 s at 95°C, 30 s at 50°C, and 90 s at 72°C; and one final step of 3 min at 72°C.

Loci that generated a single amplification product of the expected size (Table 2) were sequenced in both directions using 1 µl of the PCR product, BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Inc.) and 0.16 µM primer in a 20-µl reaction volume. Sequencing products were run on an ABI 3130 *xl* Genetic Analyzer (Life Technologies Inc.). Electropherograms were inspected visually to ensure proper base calling. Nucleotide positions that showed double peaks were scored as heterozygous. Sequences were aligned using BioEdit, version 7.2.0 (Hall 1999). Loci that failed to produce a single PCR product, contained indels, contained intralineage mutations, or were not variable were not investigated further.

For all successfully sequenced loci, a DNA sequence alignment that included samples of all dominant Canadian genotypes and samples of other *P. infestans* genotypes (Table 1) was used for SNP mining. SNP were considered candidates for ASO primer design if they distinguished one or more of the dominant Canadian genotypes. For each candidate SNP, a common primer and two ASO primers were designed. For a given SNP, ASO primers are identical except for the last base at the 3′ position. Primer selection criteria included a melting temperature of 66°C, primer lengths of 18 to 22 bp, avoidance of secondary structures, and PCR products of less than 200 bp.

Optimization of ASO primer pairs. The real-time PCR conditions of the ASO primer pairs were optimized using two isolates of each dominant *P. infestans* Canadian genotype (US-8, US-11, US-22, US-23, and US-24) and a negative control template (Table 1). For each SNP tested, two separate PCR reactions were

Table 3. Primers and standard curve statistics for nine optimized allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) assays developed for *Phytophthora infestans*

Name	Primer sequence[a]	T_a (°C)[b]	$y = ax + y_0$[c]	R^2	Efficiency	LOD (fg/µl)[d]
ASO Arp23-182 A[e]	GACGCTGTATTTGCTGAGATTA	68	$y = -3.42x + 18.71$	0.994	0.96	500
ASO Arp23-182 T[e]	GACGCTGTATTTGCTGAGATTT	…	$y = -3.05x + 19.76$	0.987	1.13	…
RevCommonArp23-207[f]	CCCAAACACCAGAATACACGAGTTAGC	…	…	…	…	…
ASO PUA-120 T[e]	GTTGCCAAATGGTTAGTTATCT	66	$y = -3.72x + 21.63$	0.986	0.86	50
ASO PUA-120 A[e]	GTTGCCAAATGGTTAGTTATCA	…	$y = -3.47x + 23.06$	0.983	0.94	…
RevCommonPUA-190[f]	GTGCAACACGATCTGGATGTGG	…	…	…	…	…
ASO PUA-225 C[e]	CACGAAGGCGAGCCTC	64	$y = -3.25x + 18.32$	0.994	1.03	50
ASO PUA-225 T[e]	CACGAAGGCGAGCCTT	…	$y = -3.28x + 21.20$	0.997	1.02	…
RevCommonPUA-260[f]	GCAGCAGCTTGAGCGTGG	…	…	…	…	…
ASO PUA-570 G[e]	CATGTACGTGGTGTAATTATTTG	64	$y = -2.99x + 19.11$	0.987	1.16	50
ASO PUA-570 A[e]	CATGTACGTGGTGTAATTATTTA	…	$y = -3.29x + 21.03$	0.982	1.01	…
RevCommonPUA-601[f]	TCTTGTTGCGACTGACAGAAACAT	…	…	…	…	…
Rev-ASO Ras-98 C(G)[f]	CAATGTAATGGCAATCTAGACC	64	$y = -3.28x + 18.78$	0.992	1.02	50
Rev-ASO Ras-98 A(T)[f]	CAATGTAATGGCAATCTAGACA	…	$y = -3.29x + 19.64$	0.982	1.01	…
CommonRas-41[e]	CATCGGTGTTGACTTTGTGAGTGT	…	…	…	…	…
ASO Ras-376 T[e]	GGACCAGGAGTCGTTCAAT	66	$y = -3.14x + 17.10$	0.998	1.08	50
ASO Ras-376 C[e]	GGACCAGGAGTCGTTCAAC	…	$y = -3.33x + 17.47$	0.990	1.00	…
RevCommonRas-468[f]	TCACAGGCGTATCTGCAAATCAAAGC	…	…	…	…	…
RevASO 3332F-225 C(G)[f]	CCCTCGTGGGCTTCAC	64	$y = -3.33x + 20.15$	0.982	1.00	500
RevASO 3332F-225 G(C)[f]	CCCTCGTGGGCTTCAG	…	$y = -3.64x + 22.29$	0.996	0.88	…
Common3332F-225[e]	CAAAGCGTGCACAGGTAACGTG	…	…	…	…	…
RevASO 3332F-258 G(C)[f,g]	TGGACCGGATCTTCGG	64	$y = -3.41x + 18.01$	0.991	0.96	500
RevASO 3332F-258 T(A)[f,g]	TGGACCGGATCTTCGT	…	$y = -3.61x + 19.67$	0.990	0.89	…
RevASO 3332F-344 G(C)[f]	CTAAGCAATGCACGCTTG	60	$y = -3.36x + 17.24$	0.996	0.98	500
RevASO 3332F-344 A(T)[f]	CTAAGCAATGCACGCTTA	…	$y = -3.49x + 20.34$	0.997	0.93	…
Common3332F-344[e]	CGGTCCACGAAGACGGA	…	…	…	…	…

[a] Single-nucleotide polymorphisms (SNP) are underlined.
[b] Annealing temperature.
[c] Standard curve linear equation (a = slope, y_0 = intercept at origin) to calculate the efficiency of the PCR.
[d] LOD = limit of detection.
[e] Forward primer.
[f] Reverse primer.
[g] Primers used with the common primer Common3332F-225.

Table 4. Expected genotypes of the five dominant Canadian genotypes of *Phytophthora infestans* at nine optimized allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) assays

Genotype	ASO Arp23-182	ASO PUA-120	ASO PUA-225	ASO PUA-570[a]	RevASO Ras-98	ASO Ras-376	RevASO 3332F-225	RevASO 3332F-258	RevASO 3332F-344
US-8	AA	TT	CC	AA	GG	TC	GG	CC	CT[b]
US-11	AA	TT	CC	GG	GT[b]	TC	GG	CA[b]	CC
US-22	AT[b]	TT	CC	GA	GT[b]	TT[b]	GG	CC	CC
US-23	AT[b]	TA[b]	CT[b]	GA	GG	TC	GC[b]	CC	CC
US-24	AA	TT	CC	AA	GG	TC	GC[b]	CC	CC

[a] The three genotypes at this ASO-PCR assay are diagnostic of *P. infestans* genotypes.
[b] Indicates the diagnostic genotypes at each of the ASO-PCR assays.

conducted, one for each allele targeted. All reactions were carried out in 25-µl volumes with the following final concentrations: 1× of GoTaq qPCR Master Mix (Promega Corp.), 0.3 µM each primer, and approximately 1 ng of template DNA. PCR conditions were as follows: 3 min at 95°C; 45 cycles of 15 s at 94°C, 30 s at an annealing temperature between 60 and 68°C (2°C increment per test), and 30 s at 72°C; and 15 s at 95°C followed by a melting curve from 55 to 95°C, with a reading every 1.0°C and a hold for a 6-s measurement. Fluorescence was also measured during the extension phase at 72°C. All reactions were run on a Rotor-Gene 6000 Real-Time PCR machine (Corbett-Research, Montreal Biotech Inc., Dorval, QC, Canada) and data were analyzed with Rotor-Gene Q series software (version 1.7; Corbett-Research).

Validation of ASO primer pairs. To distinguish and identify all five dominant Canadian genotypes of *P. infestans*, a combination of nine ASO-PCR assays was validated using a blind test (Table 3) performed at the Canadian Food Inspection Agency (Ottawa, ON, Canada). The blind test consisted of 31 *Phytophthora* samples and included one negative control template, two to six isolates of each of the dominant Canadian *P. infestans* genotypes (US-8, US-11, US-22, US-23, and US-24), three other *P. infestans* genotypes (US-1, US-6, and US-7), as well as eight other *Phytophthora* spp. (Table 1). DNA concentrations ranged from ≤0.03 to 1.0 ng/µl. Samples were tested with the nine optimized ASO primer pairs, and an overall profile was established for each sample based on the genotypes and profiles obtained at each locus. For each of the loci tested, PCR assays were performed at the optimal annealing temperatures listed in Table 3, and data analyses were as described above.

Recombinant isolates from Canada and the eastern United States also were used to validate further the nine optimized ASO-PCR assays. In all, 17 recombinant *P. infestans* isolates and one negative control template were included in the test (Table 1). DNA concentrations ranged from 0.8 to 1.2 ng/µl. Samples were tested with the nine optimized ASO primer pairs, and an overall profile was established for each sample based on the genotypes and profiles obtained at each locus. For each of the loci tested, PCR assays were performed at the optimal annealing temperatures listed in Table 3. Reactions were run on either a Rotor-Gene 6000 real-time PCR machine or a StepOne Real-Time PCR System. Data were analyzed with either Rotor-Gene Q series software, version 1.7 or StepOne Software, version 2.3.

Standard curves of ASO primer pairs. Serial dilutions of *P. infestans* DNA were used to produce standard curves for each targeted allele of each of the nine optimized ASO primer pairs to determine the limit of detection and sensitivity of the assays (Table 3). DNA serial dilutions consisted of one *P. infestans* isolate heterozygous for a specific primer pair (different isolates were used for the different primer pairs; Table 1) diluted in a series of 1:10 from

Table 5. Cycle threshold (Ct) values, genotypes, and overall profile for a blind panel of *Phytophthora infestans* and other *Phytophthora* spp. at each of nine allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) assays[a]

				ASO Arp23-182			ASO PUA-120			ASO PUA-225			ASO PUA-570		
Sample	Isolate	Species	Genotype	A	T	Type[b]	T	A	Type	C	T	Type	G	A	Type
1	Pi-08	*Phytophthora infestans*	US-8	20.9	32.4[d]	AA	21.6	–	TT	21.5	30.7	CC	37.6	22.6	AA
2	Pi-04	*P. infestans*	US-11	21.7	–	AA	21.6	38.6[d]	TT	22.1	31.9[d]	CC	22.4	34.9	GG
3	Pi-03	*P. infestans*	US-23	22.7	23.5	AT[e]	22.6	23.7	TA[e]	22.9	24.9	CT[e]	24.5	24.0	GA
4	LRC-9	*P. infestans*	US-23	23.8	24.4	AT[e]	23.3	24.3	TA[e]	23.5	25.4	CT[e]	25.0	25.0	GA
5	LRC-2	*P. infestans*	US-8	25.5	40.0[d]	AA	24.8	36.2[d]	TT	25.4	35.4[d]	CC	33.6[d]	26.6	AA
6	LRC-3	*P. infestans*	US-8	24.8	–	AA	24.5	–	TT	24.9	33.7	CC	39.8	26.2	AA
7	P10106	*P. infestans*	US-7	27.3	–	AA	26.6	–	TT	27.2	36.2[d]	CC	–	28.5	AA
8	Pi-13	*P. infestans*	US-24	22.6	–	AA	22.1	40.3[d]	TT	22.6	31.8[d]	CC	32.8	24.0	AA
9	Pi-12	*P. infestans*	US-22	21.5	21.3	AT[e]	20.2	35.5[d]	TT	20.5	30.6[d]	CC	22.0	23.0	GA
10	Pi-14	*P. infestans*	US-24	22.8	34.4[d]	AA	22.2	38.9[d]	TT	22.9	33.4[d]	CC	**30.6**	**24.3**	**AA**
11	Pi-05	*P. infestans*	US-11	21.8	–	AA	21.8	32.2[d]	TT	22.0	31.5[d]	CC	22.4	44.4[d]	GG
12	1351798	*P. pseudosyringae*	N/A	–	–	N/A	–	39.4[d]	N/A	30.7[d]	34.2[d]	N/A	–	–	N/A
13	BR 675	*P. cactorum*	N/A	–	38.3[d]	N/A	–	–	N/A	27.0[d]	27.2[d]	N/A	–	–	N/A
14	LRC-5	*P. infestans*	US-11	27.3	32.3[d]	AA	26.0	–	TT	26.4	38.5[d]	CC	25.9	–	GG
15	P1296	*P. infestans*	US-1	25.3	35.3[d]	AA	27.6	27.0	TA[e]	27.5	27.0	CT[e]	26.7	–	GG
16	Pi-06	*P. infestans*	US-11	21.9	–	AA	21.7	36.7[d]	TT	21.9	31.0	CC	22.4	42.8[d]	GG
17	Pi-10	*P. infestans*	US-22	21.2	21.0	AT[e]	20.1	35.2[d]	TT	20.3	30.5[d]	CC	21.7	22.7	GA
18	Pi-09	*P. infestans*	US-8	21.1	40.0[d]	AA	21.2	39.1[d]	TT	21.6	31.3[d]	CC	34.4	23.2	AA
19	P8372	*P. infestans*	US-6	25.7	–	AA	25.3	–	TT	25.2	38.4[d]	CC	26.2	–	GG
20	BR 255	*P. nicotianae*	N/A	–	33.3[d]	N/A	32.7[d]	–	N/A	31.0[d]	34.2[d]	N/A	–	–	N/A
21	BR 529	*P. megasperma*	N/A	–	32.4[d]	N/A	–	–	N/A	33.4[d]	29.2[d]	N/A	–	–	N/A
22	LRC-8	*P. infestans*	US-23	25.6	25.9	AT[e]	24.5	25.2	TA[e]	25.6	27.6	CT[e]	26.1	26.1	GA
23	Pi-01	*P. infestans*	US-23	22.7	23.8	AT[e]	22.2	23.9	TA[e]	23.0	24.8	CT[e]	24.3	23.9	GA
24	Pi-07	*P. infestans*	US-8	21.0	–	AA	21.1	35.6[d]	TT	21.5	31.4	CC	33.3	23.0	AA
25	LRC-6	*P. infestans*	US-11	24.5	–	AA	24.6	–	TT	24.9	34.7[d]	CC	24.8	–	GG
26	LRC-1	*P. infestans*	US-8	26.1	32.1[d]	AA	24.9	–	TT	26.1	33.4	CC	40.7	26.5	AA
27	DAOM 236393	*P. quercina*	N/A	–	–	N/A	–	–	N/A	34.4[d]	36.9[d]	N/A	–	–	N/A
28	P3009	*P. mirabilis*	N/A	–	–	N/A	32.9[d]	–	N/A	31.1	38.4[d]	CC	–	–	N/A
29	BR 518	*P. citricola*	N/A	–	–	N/A	–	–	N/A	34.2[d]	40.9[d]	N/A	–	–	N/A
30	CBS 101553	*P. ramorum*	N/A	–	–	N/A	–	33.0[d]	N/A	34.7[d]	35.3[d]	N/A	–	–	N/A
31	N/A	N/A	Negative	–	–	N/A	–	–	N/A	34.0[d]	35.8[d]	N/A	–	–	N/A

(*continued on next page*)

[a] Ct set manually at 0.05. The three genotypes at the ASO PUA-570 assay are diagnostic of *P. infestans* genotypes. Symbols: – indicates Ct > 45 and N/A = not applicable.

[b] Isolates were considered heterozygous when they displayed a difference of ≤4 Ct at a given ASO primer pair, and homozygous when they displayed a difference of ≥7 Ct, with one exception shown in bold font. This exception displayed a difference of ≥6 Ct and was considered homozygous.

[c] Nucleotide in parentheses is the complement of the target single-nucleotide polymorphism and is shown in the sense orientation.

[d] Combined analyses of Ct values and melt curves revealed nonspecific amplifications.

[e] Indicates the diagnostic genotypes at each of the ASO-PCR assays.

5 ng/µl to 5 fg/µl. Standard curves were replicated three times for each dilution for each optimized ASO primer pair.

Test of infected plant material. Infected plant material from leaf inoculation experiments performed at Agriculture and Agri-Food Canada (St-Jean-sur-Richelieu, QC, Canada) was used to test the nine optimized ASO-PCR assays developed in this study with environmental material. *P. infestans* isolates of US-23 and US-24 genotypes (PiUS23NB and Pi2011-072NB, respectively) were used to inoculate leaves of *S. tuberosum* 'Russet Burbank'. *P. infestans* isolates were cultured on Rye B agar medium (Caten and Jinks 1968) at room temperature with a natural photoperiod for 7 to 25 days until sporulation, prior to inoculation. Once sporulation occurred, distilled water was poured over the petri dishes, and the sporangia were suspended by gently agitating the petri dishes. The suspension was quickly transferred to a spray bottle for inoculation onto potato leaves. For each genotype, between 3 and 12 potato plants were inoculated by spraying the sporangia solution onto the two sides of each leaf. Inoculated plants were each covered with a plastic bag to maintain 100% relative humidity (RH). Potato plants were grown in the dark at 18°C and 100% RH for 24 h. After this period, the plastic bags were removed, the temperature was kept at 18°C, RH was adjusted to 95%, and light was adjusted to a photoperiod of 10 h/day. After 7 days, potato plants were examined for infection by *P. infestans*. For four inoculated leaves/plants, approximately three discs were cut at the leading edge of the infection site with a number 4 cork borer, and kept at –20°C until DNA extraction. DNA was extracted from infected plant material (4 discs/tube) using the DNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD) following the manufacturer's recommendations. DNA was then purified using the QuickPick SML Plant DNA magnetic particle purification kit (BioNobile, Turku, Finland) and diluted 1/100. For each of the nine optimized assays, PCR assays were performed at the optimal annealing temperatures listed in Table 3 with the PCR conditions described above. All reactions were run on a StepOne Real-Time PCR System and data were analyzed with StepOne Software, version 2.3.

Results

DNA polymorphisms. Among the 28 loci selected for PCR amplification and sequencing on the basis of sufficient primer and sequence information, five nuclear genes and eight flanking microsatellite regions generated a single amplification product, were variable, and did not contain indels (Table 2). These loci were mined for SNP among one or more of the dominant Canadian *P. infestans* genotypes. The number of SNP found at each locus and the number of SNP showing variation among one or more of the five dominant Canadian genotypes are represented in Table 2. All of the sequences produced for these loci were deposited in GenBank (Table 2).

Optimization of ASO-PCR assays. ASO primers were designed to amplify specifically the alleles of one or more dominant Canadian *P. infestans* genotypes at 14 of the 50 variable SNP identified from

Table 5. (*continued from preceding page*)

RevASO Ras-98			ASO Ras-376			RevASO 3332F-225			RevASO 3332F-258			RevASO 3332F-344			
C(G)[c]	A(T)	Type	T	C	Type	C(G)	G (C)	Type	G (C)	T(A)	Type	G (C)	A(T)	Type	Overall profile
21.5	32.4[d]	GG	20.7	21.5	TC	21.6	34.4	GG	20.9	32.7	CC	21.8	25.3	CT[e]	US-8
22.7	24.2	GT[e]	21.4	22.4	TC	22.7	43.5[d]	GG	22.3	23.2	CA[e]	25.1	34.3	CC	US-11
22.5	32.5[d]	GG	22.9	21.7	TC	23.2	26.0	GC[e]	21.9	36.5	CC	22.1	34.4	CC	US-23
22.9	30.5[d]	GG	23.1	22.2	TC	24.1	26.8	GC[e]	22.5	40.4[d]	CC	22.9	35.1	CC	US-23
25.0	28.3[d]	GG	24.5	25.9	TC	26.5	–	GG	24.3	–	CC	25.8	29.2	CT[e]	US-8
24.8	34.5[d]	GG	24.1	25.4	TC	25.6	–	GG	24.1	–	CC	25.2	29.3	CT[e]	US-8
27.3	27.8	GT[e]	25.9	32.3	TT[e]	27.7	–	GG	29.0	27.1	CA[e]	27.1	41.1[d]	CC	Other *P. infestans*
22.9	31.8[d]	GG	22.0	23.2	TC	23.6	26.3	GC[e]	22.0	31.1	CC	22.7	35.1	CC	US-24
21.7	22.0	GT[e]	19.3	28.9	TT[e]	20.8	35.6[d]	GG	20.0	40.3[d]	CC	20.7	33.5	CC	US-22
22.6	30.6[d]	GG	22.0	23.2	TC	23.9	26.4	GC[e]	22.0	–	CC	22.7	36.4	CC	US-24
22.7	24.0	GT[e]	21.3	22.3	TC	22.1	–	GG	22.0	23.1	CA[e]	22.1	35.0	CC	US-11
–	30.4[d]	N/A	–	–	N/A	26.2[d]	39.0[d]	N/A	40.2[d]	37.1[d]	N/A	36.2[d]	35.9[d]	N/A	Not *P. infestans*
39.4[d]	33.6[d]	N/A	28.3[d]	18.2[d]	N/A	17.8[d]	38.8[d]	N/A	35.3	39.9[d]	CC	31.4	38.8[d]	CC	Not *P. infestans*
26.9	28.6	GT[e]	25.8	26.9	TC	27.7	–	GG	26.0	27.5	CA[e]	25.7	41.0[d]	CC	US-11
26.3	–	GG	24.8	34.4	TT[e]	25.2	–	GG	25.1	26.2	CA[e]	25.9	35.7[d]	CC	Other *P. infestans*
22.8	24.1	GT[e]	21.1	22.4	TC	22.7	–	GG	22.0	23.1	CA[e]	22.2	34.3	CC	US-11
21.5	22.0	GT[e]	19.2	29.1	TT[e]	20.6	36.9[d]	GG	20.0	36.8[d]	CC	20.6	33.1	CC	US-22
21.5	31.2[d]	GG	20.6	21.3	TC	21.6	–	GG	20.8	–	CC	21.9	25.2	CT[e]	US-8
25.6	–	GG	25.1	24.9	TC	25.3	–	GG	24.9	–	CC	25.9	44.9[d]	CC	Other *P. infestans*
–	33.0[d]	N/A	–	–	N/A	30.2[d]	–	N/A	–	–	N/A	30.9[d]	34.5[d]	N/A	Not *P. infestans*
–	32.5[d]	N/A	33.0[d]	24.1[d]	N/A	30.0[d]	–	N/A	–	–	N/A	–	39.8[d]	N/A	Not *P. infestans*
24.1	37.1[d]	GG	24.9	23.9	TC	26.5	29.1	GC[e]	24.2	–	CC	24.7	37.3	CC	US-23
22.4	32.1[d]	GG	22.8	21.8	TC	23.6	26.5	GC[e]	21.9	37.3[d]	CC	22.6	35.2	CC	US-23
21.5	31.5[d]	GG	20.9	21.7	TC	21.7	34.2	GG	20.9	38.9[d]	CC	21.8	25.4	CT[e]	US-8
25.0	27.0	GT[e]	24.4	24.8	TC	24.9	–	GG	24.3	25.8	CA[e]	24.6	37.5	CC	US-11
25.7	30.9[d]	GG	25.3	25.8	TC	27.0	–	GG	25.2	–	CC	25.9	29.8	CT[e]	US-8
–	–	N/A	–	–	N/A	29.2[d]	–	N/A	–	–	N/A	31.7[d]	34.8[d]	N/A	Not *P. infestans*
30.9[d]	–	N/A	–	29.4	CC	34.8	–	GG	34.2	–	CC	32.7	–	CC	Not *P. infestans*
–	30.7[d]	N/A	–	–	N/A	28.9[d]	–	N/A	–	–	N/A	35.4[d]	42.5[d]	N/A	Not *P. infestans*
32.0[d]	31.2[d]	N/A	–	–	N/A	38.0[d]	–	N/A	–	–	N/A	39.3[d]	–	N/A	Not *P. infestans*
35.0[d]	36.5[d]	N/A	–	–	N/A	37.9[d]	–	N/A	–	–	N/A	–	–	N/A	Negative

the sequencing results (Table 3; Supplementary Table S2) based on each locus position in the sequence, the requirements for designing primers, and the relative abundance of SNP specific to one or more of the dominant genotypes in Canada. For the nine optimized ASO-PCR assays listed in Table 3, *P. infestans* isolates presented the expected genotypes for at least one of the annealing temperatures tested. Five ASO-PCR assays targeted only one genotype: ASO PUA-120 and -225 were specific to isolates of US-23, ASO Ras-376 was specific to isolates of US-22, RevASO 3332F-258 was specific to isolates of US-11, and RevASO 3332F-344 was specific to isolates of US-8 (Table 4). The four remaining assays targeted two or more genotypes: ASO Arp23-182 targeted US-22/US-23 genotypes, RevASO Ras-98 targeted US-11/US-22 genotypes, RevASO 3332F-225 targeted US-23/US-24 genotypes, and ASO PUA-570 targeted three genotypes (US-8/US-24, US-11, and US-22/US-23) (Table 4). The most significant cycle threshold (Ct) differences were obtained at the optimal temperatures reported in Table 3, with >7 Ct difference observed for homozygous genotypes at all loci except ASO PUA-570 (Supplementary Table S3). Melting curve analyses also showed a single peak for the nine optimized assays, indicating specific amplification at all loci (Supplementary Table S4). Therefore, for a given ASO-PCR assay, samples were considered heterozygous when the difference in Ct values between both primers was ≤4 and homozygous when the difference in Ct values was ≥7.

For the five other ASO-PCR assays listed in Supplementary Table S2, either the *P. infestans* isolates did not produce the expected genotype profile at any of the temperatures tested or the Ct differences were not large enough to discriminate homozygotes from heterozygotes. Three of these assays targeted only one genotype: RevASO Ras-136 and ASO Ras-260 were specific to isolates of US-11 while RevASO 3332-312 was specific to isolates of US-8. The two other assays each targeted three genotypes: ASO PUA-426 targeted US-8/US-24, US-11, and US-22/US-23 isolates and RevASO Trp1-136 targeted US-8/US-22, US-11/US-24, and US-23 isolates. Because these five assays gave unreliable results, they were not investigated further.

Validation of nine ASO-PCR assays. The nine optimized ASO-PCR assays revealed unique profiles for all five dominant Canadian *P. infestans* genotypes. Expected genotypes and profiles for each of the assays are listed in Table 4. No false assignments were observed in the blind tests and all Canadian genotypes were identified correctly (Table 5). The three other *P. infestans* genotypes included in the panel (US-1, US-6, and US-7) also displayed unique profiles for the nine ASO-PCR assays. These samples were identified as other *P. infestans* genotypes in the overall profile (Table 5). For most of the assays, no specific amplifications were observed for the other *Phytophthora* spp. tested, thus enabling their identification as not *P. infestans* isolates. Specific amplifications were obtained in ≤3 of the assays for *P. cactorum* and *P. mirabilis*, two species more closely related to *P. infestans* than the other *Phytophthora* spp. included in the panel (Table 5). Combined analyses of Ct values and melt curves revealed nonspecific amplifications for all negative samples that had positive Ct values (Table 5; Supplementary Table S5).

The nine optimized ASO-PCR assays also revealed unique profiles for 11 of the 17 recombinant *P. infestans* isolates tested (Table 6). These combinations of genotypes were not observed in the blind panel. A new genotype (TT) not observed previously was obtained with the ASO Arp23-182 locus for isolate US100034, and confirmed by sequencing (data not shown). As observed for the blind test, combined analyses of Ct values and melt curves revealed nonspecific amplifications for negative samples that had positive Ct values (Table 6; data not shown for melt curves).

Standard curves of ASO primer pairs. The limit of detection of the nine optimized ASO-PCR assays ranged from 50 to 500 fg/µl (Table 3) for at least one of the three replicated dilutions per assay. Efficiency ranged from 0.84 to 1.16, with R^2 from 0.98 to 0.99 (Table 3).

Test of infected plant material. DNA extracted from potato leaves infected with *P. infestans* US-24 (Table 1) presented the expected genotype for all ASO markers tested (Supplementary Table S6). In addition, DNA extracted from potato leaves infected with *P. infestans* US-23 presented the expected genotype for all markers except two (ASO Arp23-182 and RevASO 3332F-225). For the latter markers, the genotypes detected were not those expected and

Table 6. Cycle threshold (Ct) values, genotypes, and overall profile obtained for possible recombinant *Phytophthora infestans* isolates from the United States and Canada for nine optimized allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) assays[a]

		ASO Arp23-182			ASO PUA-120			ASO PUA-225			ASO PUA-570[b]			RevASO Ras-98		
Isolate	**Genotype**	**A**	**T**	**Type[c]**	**T**	**A**	**Type**	**C**	**T**	**Type**	**G**	**A**	**Type**	**C(G)[d]**	**A(T)**	**Type**
US110093	GDT-06	25.3	–	AA	20.3	40.1	TT	22.4	35.3	CC	40.0	27.3	AA	23.2	–	GG
US110074	GDT-04	26.8	25.0	AT[e]	20.2	32.8	TT	22.3	35.2	CC	40.8	27.1	AA	24.3	23.6	GT[e]
US110072	GDT-07	26.5	26.2	AT[e]	20.7	–	TT	22.7	34.9	CC	25.4	29.6	GA	23.2	–	GG
US100023	GDT-13	25.5	24.8	AT[e]	19.8	35.6	TT	22.4	34.0	CC	**24.5**	**29.6**	**GA**	22.0	42.8	GG
US110061	GDT-05	24.0	–	AA	19.4	34.9	TT	21.5	34.0	CC	24.5	28.2	GA	22.6	23.7	GT[e]
US100033	GDT-16	24.3	–	AA	19.7	35.2	TT	21.6	33.6	CC	41.0	26.9	AA	21.5	–	GG
US100034	GDT-20	–	23.2	TT	18.9	39.1	TT	21.6	34.9	CC	24.2	27.3	GA	21.6	–	GG
US110086	GDT-03	23.9	–	AA	19.3	38.8	TT	21.6	33.8	CC	24.1	27.1	GA	22.7	24.2	GT[e]
US110085	GDT-11	25.7	–	AA	20.4	–	TT	22.1	34.4	CC	**24.9**	**29.8**	**GA**	23.6	23.7	GT[e]
US100029	GDT-12	24.2	–	AA	19.5	37.6	TT	21.8	33.7	CC	**24.0**	**29.3**	**GA**	22.6	24.2	GT[e]
US100032	GDT-15	24.0	–	AA	19.8	36.7	TT	22.2	34.8	CC	39.6	28.0	AA	22.7	–	GG
US110084	GDT-01	26.9	26.8	AT[e]	21.5	37.0	TT	23.3	37.3	CC	26.4	30.2	GA	25.0	24.7	GT[e]
US100019	GDT-19	26.1	24.7	AT[e]	20.4	37.9	TT	21.9	34.5	CC	24.5	28.6	GA	22.0	42.5	GG
US110064	GDT-02	24.7	24.7	AA	20.2	37.1	TT	22.5	36.3	CC	24.7	28.8	GA	23.4	24.2	GT[e]
US110092	GDT-17	24.4	____	AA	19.8	36.9	TT	21.9	33.5	CC	39.6	28.1	AA	23.2	24.5	GT[e]
US110071	GDT-08	26.6	24.5	AT[e]	19.7	36.5	TT	21.6	33.5	CC	25.5	26.8	GA	21.8	–	GG
US110082	GDT-09	24.7	–	AA	21.3	37.1	TT	23.0	36.3	CC	43.4	29.0	AA	21.9	40.8	GG
Negative	N/A	–	–	N/A	–	–	N/A	–	42.7[f]	N/A	–	–	N/A	–	40.5[f]	N/A

(*continued on next page*)

[a] Symbols: – indicates Ct > 45 and N/A = not applicable.
[b] The three genotypes at this ASO-PCR assay are diagnostic of *P. infestans* genotypes.
[c] Isolates were considered heterozygous when they displayed a difference of ≤4 Ct at a given ASO primer pair, and homozygous when they displayed a difference of ≥7 Ct with three exceptions shown in bold font. These exceptions displayed a difference of approximately 5 Ct and were considered heterozygous.
[d] Nucleotide in parentheses is the complement of the target single-nucleotide polymorphism and is shown in the sense orientation.
[e] Indicates the diagnostic genotypes at each of the ASO-PCR assays.
[f] Combined analyses of Ct values and melt curves revealed nonspecific amplifications for the negative control samples that showed positive Ct values.

represented new mutations for this lineage, as confirmed by sequencing (data not shown). Melting curve analyses also showed a single peak, indicating specific amplification at all loci for both genotypes tested (data not shown).

Discussion

In this study, several SNP were identified in nuclear genes and flanking microsatellite regions variable among a collection of *P. infestans* genotypes. Nine of these SNP were used to develop ASO-PCR assays that enabled identification of the five most dominant genotypes present in Canada in recent years. These assays were conducted using real-time PCR assays, ensuring a high level of sensitivity without the need to start with large amounts of sample material to test. Indeed, the limit of detection of the assays ranged from 50 to 500 fg/µl, which is in the range expected for assays located in single-copy genes or regions, as shown with *P. ramorum* (Bilodeau et al. 2007b, 2009). Also, the tests performed demonstrated that this technology can be adapted easily to different real-time PCR platforms.

From the 13 loci mined for SNP in this study, 50 sites showed variation among dominant Canadian *P. infestans* genotypes. However, SNP variation was not distributed evenly among genotypes. A greater abundance of SNP was found in US-11, US-22, and US-23 genotypes than in US-8 and US-24 genotypes. Thus, many SNP were not chosen for primer design because they could not differentiate US-8 and US-24 genotypes. Although US-8 and US-24 genotypes have distinct RFLP, allozymes, and microsatellite profiles (Danies et al. 2013; Fry et al. 2013; Hu et al. 2012; Kalischuk et al. 2012; Peters et al. 2014), a neighbor-joining tree built from microsatellite markers showed that these genotypes are very close genetically compared with US-11, US-22, and US-23 genotypes (Fry et al. 2013). Even though the nine markers developed in this study distinguish all dominant Canadian *P. infestans* genotypes, development of more markers specific to US-8 and US-24 is desirable. Additional SNP distinguishing these two genotypes could be obtained by doing a comparative analysis of their transcriptomes. Indeed, these genotypes present different phenotypic characteristics such as mating type and sensitivity to mefenoxam (Danies et al. 2013; Fry et al. 2013; Hu et al. 2012; Kalischuk et al. 2012; Peters et al. 2014), differences that should be reflected in their transcriptomes.

Although limited variation was found between US-8 and US-24 genotypes, plenty of SNP were available to differentiate US-11, US-22, and US-23 genotypes. Thus, there is some redundancy in the genotypes detected by the nine optimized ASO-PCR assays. For example, US-23 genotype is detected specifically by two ASO-PCR assays, ASO PUA-120 and -225. However, this genotype can also be detected with two other assays, ASO Arp23-182 and RevASO 3332F-225. This redundancy means that not all the assays are needed to identify the five dominant Canadian *P. infestans* genotypes. A good example is provided by the new mutations found in the US-23 genotype used in the inoculation experiment for two of the ASO-PCR assays, ASO Arp23-182 and RevASO 3332F-225. This genotype was still identified as US-23 despite the mutations, based on the profile exhibited with the seven other ASO-PCR assays. For routine usage, a subset of assays can be performed first, followed by confirmation of the profiles with the other markers, as needed by end users.

The ASO-PCR assays developed in this study were designed specifically to distinguish the five *P. infestans* genotypes dominant in Canada over the last 5 to 6 years (Kalischuk et al. 2012; Kawchuk et al. 2011; Peters et al. 2014). However, this marker system based on SNP variation is universal and could easily be extended to encompass new genotypes or genotypes present in other countries, as suggested by Abbott et al. (2010). For example, the nine optimized ASO-PCR assays distinguished the US-1, US-6, and US-7 genotypes included in the blind test in this study by producing unique profiles for each. The ASO-PCR assays also provided unique profiles for 11 of 17 possibly recombinant isolates found in Canada and the eastern United States. Theoretically, these markers could detect 19,683 possible unique genotype combinations (three possible genotypes for each of nine markers = 3^9). However, the number of possible unique genotype combinations is likely to be less with these specific assays, because eight markers differentiated only two genotypes. Even with 768 possible unique genotype combinations ($2^8 \times 3$), this should still encompass most of the *P. infestans* genotypic variation, at least in regions where reproduction is clonal. For example, in North America, where *P. infestans*

Table 6. (*continued from preceding page*)

ASO Ras-376			RevASO 3332F-225			RevASO 3332F-258			RevASO 3332F-344			
T	C	Type	C(G)	G (C)	Type	G (C)	T(A)	Type	G (C)	A(T)	Type	Overall profile
22.8	22.6	TC	22.6	–	GG	18.8	–	CC	23.2	37.5	CC	Recom A
22.4	23.3	TC	22.2	40.8	GG	18.5	42.4	CC	22.9	36.9	CC	Recom B
23.2	22.8	TC	23.2	37.0	GG	19.2	–	CC	23.5	37.2	CC	Recom C
21.3	35.0	TT[e]	21.9	44.6	GG	18.2	39.0	CC	22.6	36.5	CC	Recom D
20.9	33.3	TT[e]	21.7	45.0	GG	18.0	–	CC	22.2	36.2	CC	Recom E
20.6	34.4	TT[e]	21.5	–	GG	17.8	–	CC	21.8	35.7	CC	Recom F
21.8	21.8	TC	21.4	38.3	GG	17.9	–	CC	21.9	35.8	CC	Recom G
21.5	22.1	TC	21.4	41.3	GG	17.8	42.5	CC	22.2	35.8	CC	Recom H
22.1	35.4	TT[e]	22.2	38.9	GG	18.2	42.0	CC	22.6	36.3	CC	Recom E
21.1	36.5	TT[e]	21.7	42.4	GG	18.1	45.0	CC	22.3	35.7	CC	Recom E
21.4	37.1	TT[e]	22.4	39.6	GG	18.9	43.4	CC	22.9	36.8	CC	Recom F
22.8	24.2	TC	23.4	–	GG	20.0	–	CC	24.2	39.3	CC	Recom I
22.9	22.5	TC	22.1	–	GG	18.7	–	CC	22.8	36.4	CC	Recom J
21.5	35.7	TT[e]	22.6	–	GG	18.8	–	CC	22.8	36.3	CC	Recom E
21.2	36.8	TT[e]	22.6	–	GG	18.3	–	CC	22.6	36.8	CC	Recom K
20.7	34.2	TT[e]	22.4	–	GG	18.3	–	CC	22.6	36.7	CC	Recom D
21.4	35.5	TT[e]	22.2	–	GG	18.3	39.9	CC	22.6	36.0	CC	Recom F
–	–	N/A	35.9[f]	–	N/A	–	–	N/A	–	–	N/A	N/A

reproduces clonally for the most part (Fry 2008), about 44 distinct genotypes (US-1 to US-24 and GDT-01 to GDT-20) have been reported from the United States over the last 20 years (Danies et al. 2013, 2014; Forbes et al. 1998; Goodwin et al. 1994, 1995, 1998; Hu et al. 2012; Peters et al. 2014). This marker system could also be implemented in regions where recombination is frequent, such as in Scandinavia (Brurberg et al. 2011; Sjöholm et al. 2013; Yuen and Andersson 2013). However, in these regions, more loci might be needed to increase the number of unique genotype combinations detected, which might pose a challenge for development and might not be ideal because of the increased number of assays that would be required.

The genotyping of 17 potentially recombinant *P. infestans* isolates from the eastern United States and Canada with the ASO-PCR assays revealed 11 unique profiles not observed previously, either for the five dominant *P. infestans* genotypes in Canada or for US-1, US-6, or US-7 genotypes. The variation observed for these profiles occurred mainly around the ASO-PCR assays specific to US-22 genotype, whereas the assays specific to other genotypes did not show variation. The high number of new genotype combinations observed for these markers is likely the result of segregation and suggests that US-22 might be a parental genotype for some of these isolates. This is consistent with the results of parentage exclusion analysis performed by Danies et al. (2014).

The development of ASO-PCR assays with SYBR Green technology was chosen over the design of assays with specific probes (e.g., TaqMan, minor groove binding, or lock nucleic acids) for two reasons. First, ASO-PCR assays are easier to design than hybridization probes, and second, ASO-PCR assays are of relative low cost compared with probes because they are based on SYBR Green technology. These features make development and implementation easier for end users than probe-based assays. Probe-based assays do have advantages over ASO-PCR assays in that they are usually more specific than SYBR Green assays, emitting fluorescence only when bound to a specific region of interest, not any double-stranded DNA. However, if a melting curve is included in ASO-PCR assays with SYBR Green, this can be used to check that the product amplified is specific, as demonstrated in this study. Probe-based assays do have the advantage of options for multiplexing compared with single-plex ASO-PCR assays, which was not investigated in the present study. It might be worth developing a multiplex approach to ASO-PCR assays to decrease the number of required runs and, therefore, the time required for identification of *P. infestans* genotypes. This can be crucial during late blight outbreaks. Moreover, the assays developed in this study could be modified to one or more isothermal amplification techniques. For example, the isothermal amplification technique using recombinase polymerase amplification allows multiplexing and can be used directly in the field with portable instruments, as shown by Miles et al. (2015) for the detection of *Phytophthora* spp.

Currently, potato growers are only informed about the genotypes of *P. infestans* present in their fields late in the season. This delay is mostly caused by the lack of cost-effective methods for genotype determination. An interesting characteristic of the ASO-PCR assays developed in this study is that they can be used on environmental samples because they only require limited amounts of starting material (Bilodeau et al. 2007a, 2009). This contrasts with RFLP methods that need large amounts of DNA as starting material (Cooke and Lees 2004) that must be harvested from pure cultures of the pathogen, which can take several weeks to months to produce (L. Kawchuk, personal communication). These markers represent a complement to existing genotyping methods for *P. infestans*, offering new perspectives such as rapid determination of genotypes from incoming inoculum monitored with spore samplers (Fall et al. 2014) or from first lesions observed in potato fields or on trap plants. Furthermore, Fall et al. (2015) showed that the infection efficiency of *P. infestans* sporangia varies among genotypes. Consequently, informed late blight management decisions should consider weather conditions, the amount of inoculum present, resistance of the incoming inoculum to fungicides, and virulence of the isolates. Tests performed on DNA extracted from infected plant material revealed that the nine ASO-PCR assays developed in this study allowed the detection and identification of the US-23 and US-24 genotypes on potato without interference from potato leaf material. The nine markers developed will contribute to the genomic toolbox available to assess the genetic diversity of *P. infestans* at the intraspecific level. From a disease management standpoint, the markers and assays will help to select the most appropriate fungicides to use and their application frequency to manage late blight.

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