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Evaluation of Simple Sequence Repeat (SSR) Markers Established in Europe as a Method for the Identification of Potato Varieties Grown in Canada

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Abstract The correct identification of potato varieties is crucial to maintaining the quality level of seeds produced under the Canadian Seed Potato Certification Program. During inspection of in vitro potato plant propagation centres or seed potato production field lots, morphological characteristics may not be sufficient for the identification of plantlets or tubers and therefore molecular identification is sought by inspectors for variety confirmation. With international harmonization of testing methods in mind, we proposed to evaluate further the microsatellite (SSR) markers established successfully by Reid et al. (2009, *Euphytica* 182: 239–249, 2011), using the reference potato variety DNA collection at the Canadian Food Inspection Agency (CFIA) established and currently used for Amplified Fragment Length Polymorphism (AFLP) genotyping. The SSR markers developed by the European community laboratories were successfully validated by and established within the CFIA laboratory. Most genotypes generated for a set of 34 varieties were identical between the 2 laboratories with only 3 discrepancies due to the different interpretation of the presence/absence of the alleles. When used with potato reference DNAs of the CFIA collection, the method successfully differentiated 217 varieties apart but 10 groups, which are most likely clonal variants, were not discriminated. The SSR markers were successfully used to address 5 potato variety verification requests from CFIA inspectors during field inspections for seed potato certification. The markers successfully confirmed the presence of rogue varieties in 4 of these requests, therefore fulfilling the CFIA's mandate towards stakeholders of the Canadian potato industry in preserving the quality of certified seeds.

Resumen La identificación correcta de variedades de papa es crucial para el mantenimiento del nivel de calidad de las semillas producidas bajo el Programa Canadiense de Certificación de Semilla de Papa. Durante la inspección de los centros de propagación de plantas de papa in vitro o de los lotes de campo de producción de semilla de papa, las características morfológicas pudieran ser insuficientes para la identificación de plántulas o tubérculos, y en consecuencia, se busca la identificación molecular por los inspectores para confirmación de la variedad. Con la armonización internacional de los métodos de prueba en mente, proponemos evaluar más adelante los marcadores de microsatélites (SSR) establecidos con éxito por Reid et al. (2009, *Euphytica* 182: 239–249, 2011), usando la referencia de la colección de ADN de variedades de papa en la Agencia Canadiense de Inspección de Alimentos (CFIA) establecida y actualmente en uso para el genotipo por Polimorfismo de Longitudes de Fragmentos Amplificados (AFLP). Los marcadores SSR desarrollados por los laboratorios de la comunidad Europea se validaron con éxito por y establecidos dentro del laboratorio de la CFIA. La mayoría de los genotipos generados para un juego de 34 variedades fue idéntico entre los dos laboratorios con solo tres discrepancias debido a la interpretación diferente de la presencia/ausencia de los alelos. Cuando se usaron con ADN's de referencia de papa de la colección de la CFIA, el método diferenció exitosamente 217 variedades, excepto 10 grupos, que lo más probable es que sean variantes clonales, que no fueron discriminados. Los marcadores SSR se usaron con éxito para atender solicitudes de verificación de cinco variedades de papa, de inspectores de la CFIA durante inspecciones de campo para certificación de semilla de papa. Los marcadores confirmaron exitosamente la presencia de

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falsas variedades en cuatro de estas solicitudes, satisfaciendo entonces el mandato de la CFIA hacia participantes de la industria de papa en Canadá en la preservación de la calidad de semillas certificadas.

Keywords Variety · Microsatellites · Genotyping · *Solanum tuberosum*

Introduction

Potato (*Solanum tuberosum* L.) is a very important crop worldwide. To this day, there are more than 4,500 varieties of potatoes that are cultured in over 100 countries (http://en.agrimedia.com/libpotato/shop/list.php). As the number of available varieties increases, so does the complexity of accurate varietal identification as the morphological differences between two varieties may be very subtle due the nature of the breeding methods employed; these may include marker selected breeding and a large number of backcrossings with one of the parental varieties.

The Canadian Food Inspection Agency (CFIA) is a government agency that regulates the Potato Seed Certification Program, and has been relying on molecular confirmation of potato varieties for over a decade now. Requests for potato genotyping for variety verification purposes may originate due to possible mix-up of plantlets in repositories which may occur during propagation. But mostly, requests originate during seed certification inspections where high levels of variations in a crop are suspected to be caused by rogue varieties and which therefore result in the refusal to certify the seeds of the lot. For most requests, morphological characteristics may not be sufficient for identification (plantlets and tuber) and molecular identification is then sought for confirmation of variety. Appropriate identification of the potato varieties is crucial to maintain the high level of quality of the Canadian Seed Potato Certification Program which benefits all stakeholders involved.

To respond to variety verification requests, we have developed a procedure for molecular identification of potato varieties which resulted in the generation of a reference collection of DNA extracts representing some 224 potato varieties available in Canadian repositories. Most of the reference DNAs were extracted from plantlets originating from 2 sources (155 varieties). Using this DNA collection, a reference collection of AFLP (Amplified Fragment Length Polymorphism) fingerprints representing these potato varieties was generated. In absence of characterised SSR (Simple Sequence Repeats) markers, AFLP has been a very powerful method for distinguishing varieties. However, the AFLP technique is more challenging technically than microsatellite analysis and therefore not very suitable for a diagnostic high throughput setting or for harmonization from one laboratory to another. Furthermore, testing and results analysis using AFLP as a varietal confirmation method is time consuming and expensive.

For granting Plant Breeders' Rights for a new potato variety, the applicant must demonstrate that the new variety is Distinct, Uniform and Stable for 2 years (DUS testing) as per the International Union for the Protection of New Varieties of Plants guidelines (UPOV document TG/1/3: http://www.upov.int/en/publications/tg-rom/tg001/tg_1_3.pdf). In Europe, DUS testing is conducted by DUS testing stations as morphological and physiological characteristics of the new variety are assessed against other well characterised varieties. However, the increasing volume of varieties to test and the maintenance of germplasm necessary to conduct DUS testing is becoming increasingly challenging. Therefore, a UPOV working group on Biochemical and Molecular Techniques, and DNA-Profiling in Particular (BMT) was created and tasked with consideration of the potential use of molecular markers in DUS testing. In 2006, the Community Plant Variety Office of the European Community (CPVO) funded a project with the purpose of constructing an integrated database that would include microsatellite genotypes and morphological characteristics specific to potato varieties in the European Union Common Catalogue. The four partners involved were organisations responsible for DUS testing in Germany, the Netherlands, Poland and United Kingdom. Upon completion of this project, approximately 1,000 varieties of potato were collected and genotyped using 9 microsatellite (simple sequence repeats; SSR) markers (Reid et al. 2009, 2011). The results of these studies show that nearly all varieties (99.5 %) had a unique genotype except for some mutants (Reid et al. 2011). The two laboratories involved in the genotyping analysis, scored identical genotypes for almost all varieties. The few differences were usually linked to different interpretation of the presence/absence of a small number of alleles (Reid et al. 2011).

With international harmonization of testing methods in mind, we proposed to evaluate further the markers established successfully by the European group, using the reference potato variety DNA collection at CFIA. The data obtained from this project provided UPOV with scientific information fulfilling the efforts towards establishing international guidelines regarding the management and harmonisation of datasets of molecular information. Furthermore it is shown that the use of two microsatellite genotype datasets, representing potato varieties cultivated in Canada and the existing dataset in Europe, as reference instead of live material significantly improved accuracy and turnaround time for varietal verification testing. In summary, the SSR markers provide an established and improved potato variety identification method for the delivery of diagnostic results in a timely manner in support of the Canadian Seed Certification Program process.

Materials and Methods

Plant Materials and DNA Extractions

Potato in vitro cultures (plantlets) representative of varieties publicly available from culture in Canada were obtained from PhytoDiagnostics Company (British Columbia, Canada) and the Plant Propagation Centre in Fredericton (New Brunswick, Canada). Each source provided two plantlets representative of a variety. Occasionally, samples representative of varieties missing from our collection were provided by other sources as plantlet, tuber or leaflet from mature plants. For each of the potato variety tested, DNA was extracted from in vitro plantlet, tuber or leaflet (approximately 0.1 g of material) using the CTAB method (Doyle and Doyle 1990). Recently acquired varieties were extracted using the Qiagen DNeasy kit (Qiagen, USA) as per the supplier's protocol. Currently, the DNA collection contains 145 varieties originating from both cited sources and 65 varieties originating from either repository. The DNA collection acquired 14 more varieties from other miscellaneous sources for a grand total of DNA extract representative of 224 potato varieties.

Markers Sets and Amplification Conditions

The method by Reid et al. (2009, 2011) is based on the use of three multiplexed microsatellite (SSR) markers targeting 9 loci and identified as set 1: STM0019; STM3009; SSR1, set 2: STM2005; STM3012; STM3023 and set 3: STM2028; STM5136; STM5148 (Ghislain et al. 2004; Kawchuk et al. 1996; Milbourne et al. 1998). Primer sequences are described in Table 1. These markers were sufficiently robust, consistent and could differentiate more than 1,000 varieties maintained in the 4 DUS stations in Europe (Reid et al. 2009). DNA extracts representing all varieties of our collection were genotyped essentially as described by Reid et al. (2009) with some modifications. For each 10 μL reaction, 1.0 μL of 10 ng/μL suspension of DNA was amplified using 0.05 unit of Qiagen HotStarTaq Plus DNA polymerase (Qiagen, U.S.) in 1X of the buffer supplied with the enzyme. The final concentration of $MgCl_2$ was 1.75 mM, 250 μM each of the dNTPs and the primer concentration for each set is outlined in Table 1. Amplifications were performed in a PTC-200 DNA Engine Peltier thermocycler (Bio-Rad, Canada) starting with an initial denaturation at 95 °C for 5 min then followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C and a last final cycle for 10 min at 72 °C.

Calibration of Allele Scoring and Result Analysis

All PCR samples were analyzed using a Genetic Analyser ABI 3130 (Applied Biosystems, U.S.). A volume of 1.0 μL the PCR amplicons in 8.7 μL of HI-DI formamide and 0.3 μL of GeneScan™ 500 LIZ size standard (Applied Biosystems, U.S.) were run on POP-6™ though a 50 cm array. Analysis of SSR peak patterns obtained was done using GeneMapper V3.5. As described in Reid et al. (2011), alleles were assigned

Table 1 Microsatellite primer concentration for each set

Primer set	SSR marker	Number of alleles per marker	Forward and reverse primer sequences (5′-3′)[a]	Final concentration of each primer (μM)	Final concentration of primer per set (μM)
1	STM0019	10	**VIC**-AATAGGTGTACTGACTCTCAATG GTTTGAAGTAAAAGTCCTAGTATGTG	0.165	0.562
	STM3009	6	**NED**-TCAGCTGAACGACCACTGTTC GTTTGATTTCACCAAGCATGGAAGTC	0.077	
	SSR1	9	**6FAM**-GATGAGATGAGATATGAAACAACG GTTTCGCAATTCTCTTGACACGTGTCACTGAAAC	0.039	
2	STM2005	13	**NED**-TTTAAGTTCTCAGTTCTGCAGGG GTTTGTCATAACCTTTACCATTGCTGGG	0.05	0.36
	STM3012	7	**6FAM**-CAACTCAAACCAGAAGGCAAA GTTTGAGAAATGGGCACAAAAAACA	0.07	
	STM3023	4	**VIC**-AAGCTGTTACTTCATTGCTGCA GTTCTGGCATTTCCATCTAGAGA	0.06	
3	STM2028	10	**NED**-TCTCACCAGCCGGAACAT GTTTAAGCTGCGGAAGTGATTTTG	0.125	0.65
	STM5136	18	**VIC**-GGGAAAAGGAAAAGCTCAA GTTTATATGAACCACCTCAGGCAC	0.05	
	STM5148	14	**6FAM**-TCTTCTTGATGACAGCTTCG GTTTACCTCAGATAGTTGCCATGTCA	0.15	

[a] The name of the fluorescent label is indicated on the forward primer in bold

Table 2 Potato genotypes produced by both laboratories for the calibration of allele calling

Variety code	Laboratory	STM0019[a]	STM3009	SSR1	STM2005	STM3012	STM3023	STM2028	STM5136	STM5148
1	U.K.	*FH*[b]	*G*	DFI	ADF	BC	BD	AC	EFH	IJO
	Can.	*F*	*DG*	DFI	ADF	BC	BD	AC	EFH	IJO
2	U.K.	FG	FG	DL	BDF	BC	AD	BC	FH	IJO
	Can.	FG	FG	DL	BDF	BC	AD	BC	FH	IJO
3	U.K.	BFG	FG	ADI	AD	BCD	ABD	ABC	EF	IJO
	Can.	BFG	FG	ADI	AD	BCD	ABD	ABC	EF	IJO
4	U.K.	FG	BFG	DI	ABF	BF	A	AC	EH	IJO
	Can.	FG	BFG	DI	ABF	BF	A	AC	EH	IJO
5	U.K.	FG	BFG	DI	BF	B	ABD	C	CDE	IJ
	Can.	FG	BFG	DI	BF	B	ABD	C	CDE	IJ
6	U.K.	F	G	DFI	DF	BC	A	BCF	EFH	FJO
	Can.	F	G	DFI	DF	BC	A	BCF	EFH	FJO
7	U.K.	DF	BFG	ACFI	BDF	BD	AB	BC	EF	IJP
	Can.	DF	BFG	ACFI	BDF	BD	AB	BC	EF	IJP
8	U.K.	*EG*	G	DI	ABD	BC	ABD	C	FH	IJ
	Can.	*G*	G	DI	ABD	BC	ABD	C	FH	IJ
9	U.K.	*BDG*	*BFG*	*DI*	*AD*	*BCD*	*ACD*	*ACEG*	*DFH*	*JO*
	Can.	*BF*	*FG*	*DFI*	*AB*	*BF*	*AD*	*ABC*	*CEF*	*AIJN*
10	U.K.	DF	*FG*	CDI	ADF	BC	AB	C	EF	AIJ
	Can.	DF	*DFG*	CDI	ADF	BC	AB	C	EF	AIJ
11	U.K.	F	FG	DI	BDF	BC	BD	ACF	FH	IJO
	Can.	F	FG	DI	BDF	BC	BD	ACF	FH	IJO
12	U.K.	BFG	FG	ADFI	ABD	BC	AD	AC	EFH	BIJ
	Can.	BFG	FG	ADFI	ABD	BC	AD	AC	EFH	BIJ
13	U.K.	DFG	BG	DI	ABD	BCF	AB	ABC	CEH	IJP
	Can.	DFG	BG	DI	ABD	BCF	AB	ABC	CEH	IJP
14	U.K.	F	BFG	DF	ABF	B	AB	C	CF	JOP
	Can.	F	BFG	DF	ABF	B	AB	C	CF	JOP
15	U.K.	F	BFGI	CDF	BDF	B	AD	BCF	CEFH	FIO
	Can.	F	BFGI	CDF	BDF	B	AD	BCF	CEFH	FIO
16	U.K.	*BD*	*FG*	*DF*	*ABD*	*BEF*	*ACD*	*CG*	*CFH*	*JOP*
	Can.	*F*	*FG*	*DI*	*BD*	*BCF*	*ABD*	*C*	*EFH*	*IN*
17	U.K.	BF	*BG*	DFI	ABD	B	ABCD	AC	FH	IJ
	Can.	BF	*BDG*	DFI	ABD	B	ABCD	AC	FH	IJ
18	U.K.	F	G	DFI	BD	B	ABD	AC	EF	IJO
	Can.	F	G	DFI	BD	B	ABD	AC	EF	IJO
19	U.K.	*BE*	*FG*	DFI	B	BF	AD	ABC	CEFH	IJO
	Can.	*B*	*DFG*	DFI	B	BF	AD	ABC	CEFH	IJO
20	U.K.	BF	BFGK	CDFI	BD	B	AB	AC	EFH	FIJO
	Can.	BF	BFGK	CDFI	BD	B	AB	AC	EFH	FIJO
21	U.K.	BF	FG	DFI	BDF	B	BD	ABC	EFH	IJO
	Can.	BF	FG	DFI	BDF	B	BD	ABC	EFH	IJO
22	U.K.	NULL	FG	ADFI	ABF	BC	ABD	AC	EFH	IJO
	Can.	NULL	FG	ADFI	ABF	BC	ABD	AC	EFH	IJO
23	U.K.	*D*	*G*	*DGI*	*ABF*	*BC*	*ABD*	*ABC*	*CEF*	*GJ*
	Can.	*F*	*FG*	*DI*	*D*	*BF*	*ABD*	*AC*	*EF*	*IJ*
24	U.K.	D	BG	DFI	B	BC	ABD	ABC	CEF	IJO
	Can.	D	BG	DFI	B	BC	ABD	ABC	CEF	IJO
25	U.K.	F	G	DFI	ABD	BC	AD	AC	FH	IJ

Table 2 (continued)

Variety code	Laboratory	STM0019[a]	STM3009	SSR1	STM2005	STM3012	STM3023	STM2028	STM5136	STM5148
	Can.	F	G	DFI	ABD	BC	AD	AC	FH	IJ
26	U.K.	DG	BFG	DI	ABDF	BCF	AB	AC	FH	AIJP
	Can.	DG	BFG	DI	ABDF	BCF	AB	AC	FH	AIJP
27	U.K.	NULL	G	DFI	BD	BC	AB	BC	CF	AIJ
	Can.	NULL	G	DFI	BD	BC	AB	BC	CF	AIJ
28	U.K.	FG	BFG	CDI	ADF	BF	AB	AC	CFH	IJ
	Can.	FG	BFG	CDI	ADF	BF	AB	AC	CFH	IJ
29	U.K.	*B*	*FG*	*DFI*	*AB*	*BF*	*BD*	*AE*	*EH*	*BO*
	Can.	*NULL*	*FGI*	*DI*	*D*	*B*	*AD*	CF	*FH*	*FIJ*
30	U.K.	BF	FG	DI	ABDF	BF	ABD	C	CDFH	IJO
	Can.	BF	FG	DI	ABDF	BF	ABD	C	CDFH	IJO
31	U.K.	DF	*BG*	DFI	BF	BC	AC	CE	EFH	IJ
	Can.	DF	*BDG*	DFI	BF	BC	AC	CE	EFH	IJ
32	U.K.	B	FG	DF	BDF	BCF	AB	AC	CFH	IOP
	Can.	B	FG	DF	BDF	BCF	AB	AC	CFH	IOP
33	U.K.	DF	FG	CDF	BD	B	AB	C	CFH	IOP
	Can.	DF	FG	CDF	BD	B	AB	C	CFH	IOP
34	U.K.	F	FG	CDFI	ABDF	B	AD	C	CFH	IJO
	Can.	F	FG	CDFI	ABDF	B	AD	C	CFH	IJO

[a] SSR marker name

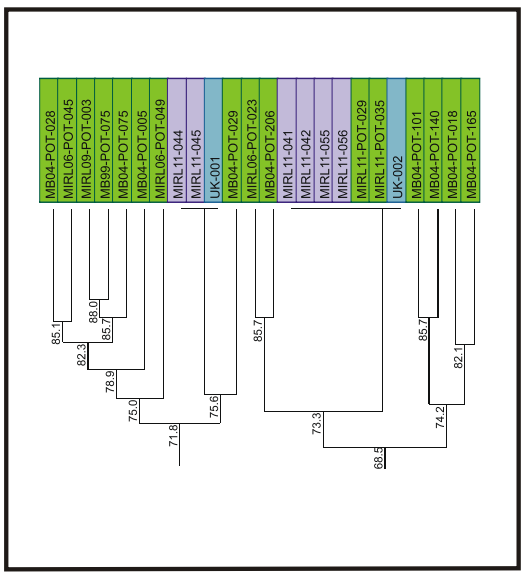
[b] Italic areas presents allele differences

letters and not numbered by base pair length which compensated for inter-laboratory reagents, instrument and human variations. To calibrate the allele scoring, a selected set of potato varieties with known genotypes were run and used to harmonize peak calling for each marker (Reid et al. 2009). Once the calibration was completed, DNA extracts from our collection (CFIA) representative of 224 potato varieties were genotyped. The 91 possible alleles were scored for presence or absence in a binary format and imported from an Access database into BioNumerics (Applied Maths, U.S.) for analysis. To assess varietal genotype similarity, the Dice correlation similarity index was determined using the BioNumerics v6.5 software and a dendogram generated using the Unweighted Pair Group Arithmetic Average (UPGMA) method.

Results

Transfer and Harmonization of the European Microsatellite Marker Method

To validate the transferred method in our laboratory, a set of 34 potato varieties common to our DNA collection and the European collection, were tested using the 3 multiplexed marker sets. The genotypes obtained were compared to those from the European genotype collection (Table 2). The genotypes shown in Table 2, matched quite closely for each variety tested except for 4 pairs; varieties 9, 16, 23 and 29. Of these 4 non-matching pairs, 2 pairs were explained by the fact that the two reference varieties tested had the same variety name but were in fact two different varieties. The discrepancies observed in the other two pairs could not be resolved but may have been due to mislabelling. Analysing and comparing the genotyping data further, of the 91 possible alleles, calling differences between the two labs were found to occur in 3 alleles spread over 2 markers. The two laboratories have been assessing presence/absence of allele E and H for marker STM0019 and D for STM3009 differently. These occasional discrepancies were due to the different interpretation of the presence/absence of the alleles. DNA quality, age or the extraction protocol were not found to be a factor, as DNA extracted from fresh material using another method, showed no significant impact on allele relative intensity and subsequent calling (data not shown). Thus, the difference in allele calling between the two groups appears to be due to differences in the reagents/machines and their impact on allele preference for amplification. To resolve these discrepancies, some of the calling rules were modified to match the European group. However as seen in Fig. 1, 4 varieties still showed one allele difference between the European and CFIA groups but nevertheless, remained segregated together.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MIRL06-POT-045
MIRL09-POT-003
MB99-POT-075
MB04-POT-075
MB04-POT-005
MIRL06-POT-049
MIRL11-044
MIRL11-045
UK-001
MB04-POT-029
MIRL06-POT-023
MB04-POT-206
MIRL11-041
MIRL11-042
MIRL11-055
MIRL11-056
MIRL11-POT-029
MIRL11-POT-035
UK-002
MB04-POT-101
MB04-POT-140
MB04-POT-018
MB04-POT-165
85.1
82.3
88.0
85.7
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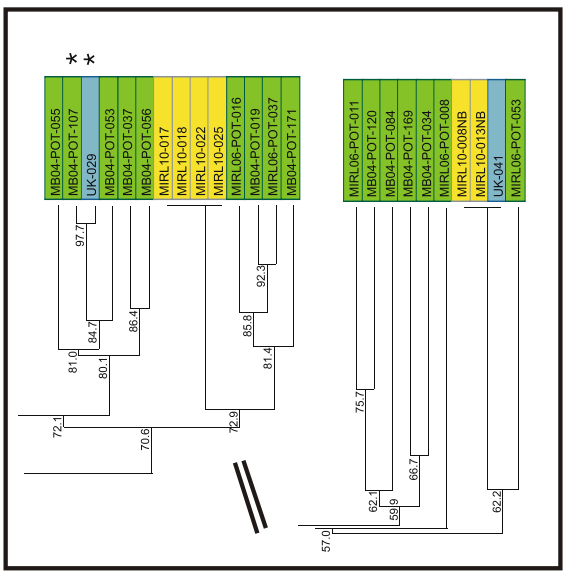
71.8
75.6
85.7
73.3
68.5
85.7
74.2
82.1

Case 1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UK-029
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MB04-POT-056
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MIRL10-018
MIRL10-022
MIRL10-025
MIRL06-POT-016
MB04-POT-019
MIRL06-POT-037
MB04-POT-171
MIRL06-POT-011
MB04-POT-120
MB04-POT-084
MB04-POT-169
MB04-POT-034
MIRL06-POT-008
MIRL10-008NB
MIRL10-013NB
UK-041
MIRL06-POT-053
97.7
81.0
84.7
80.1
86.4
72.1
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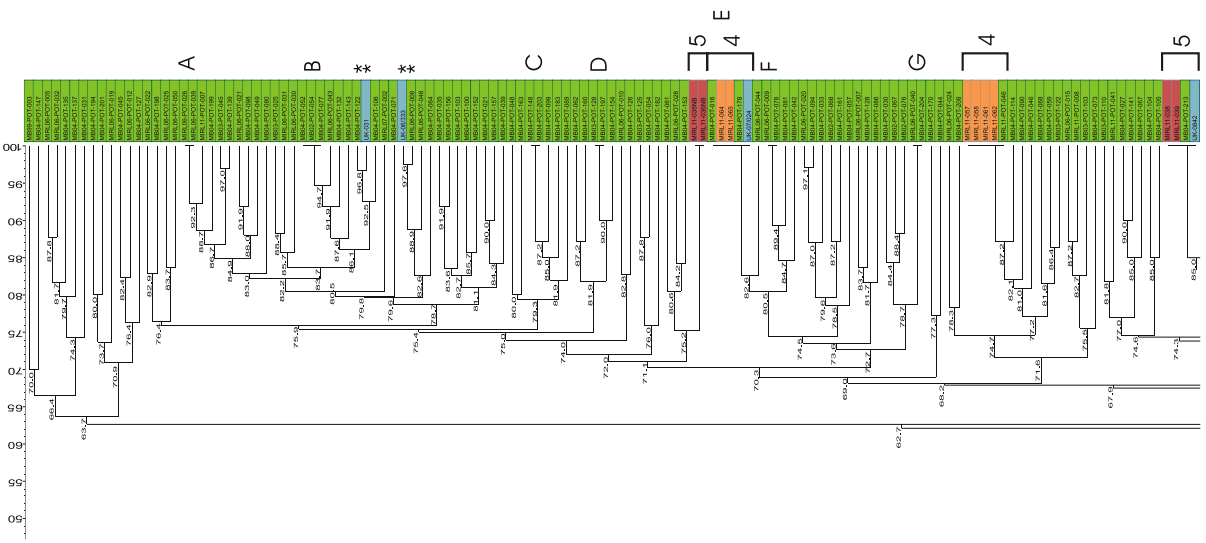
62.1
59.9
66.7
57.0
62.2

Case 2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B
C
D
E
F
G
5
4
4
5

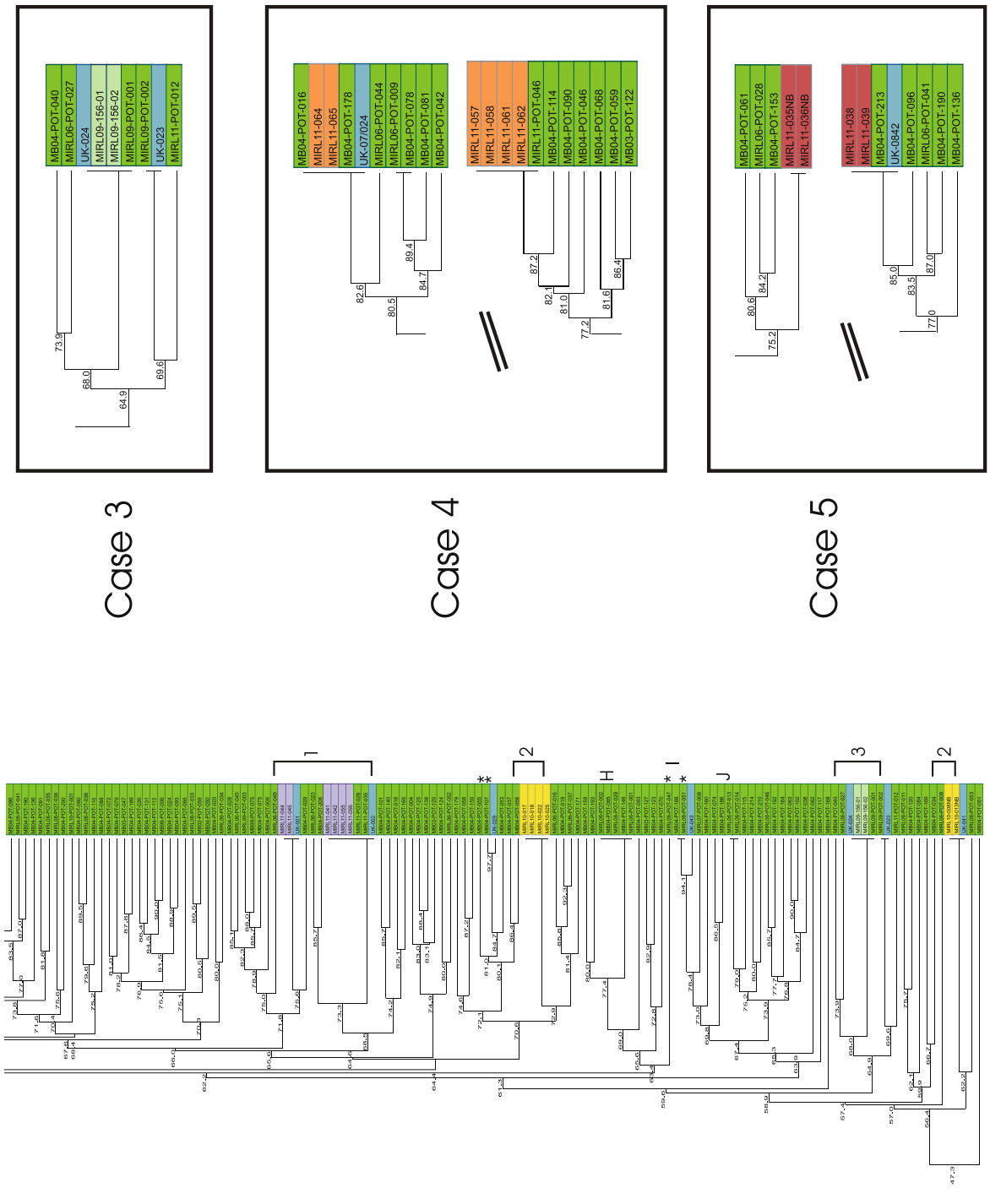

Fig. 1 UPGMA dendogram obtained using 9 microsatellite markers on the Canadian potato reference DNA collections (blocked in *green*) and samples submitted by inspectors (blocked in colour other than *green and blue*). The Dice correlation similarity index is indicated at the *top* and at *each node* in percentage. Genotypes named starting with "UK-" (blocked in *blue*) are reference from the European database. The symbol "*asterisk*" is used to indicate the 4 paired varieties for which one allele difference was observed between the European and Canadian laboratories. *Letters on the right of the dendogram* represents the 10 groups of potato varieties for which genotypes are identical (100 %). *Numbers 1 to 5* on the right of the dendogram identify the 5 potato variety verification cases. Each of these cases is enlarged and boxed on the right and labelled with the corresponding case number

Analysis of the 224 Reference Potato Varieties

Once the method using the 3 sets of multiplexed markers was calibrated, the genotyping of some 524 DNA extracts representing 224 potato varieties that are cultivated or have been cultivated in Canada, proceeded. The results are shown in Fig. 1. Potato genotypes originating from two sources or more that are identical are shown as one entry in Fig. 1. The 9 microsatellite markers differentiated all of the reference potato varieties in our collection apart from 10 groups labelled A to J in Fig. 1. Some 200 varieties were clearly differentiated with a unique genotype. 73.5 % (147/200) of the unique reference genotype patterns were confirmed by material from a second source (data not shown). Most potato variety genotype unique patterns are distinct from each other by the presence and/or absence of 3 alleles or more with a Dice similarity index of 92.3 % and below. However, there are 3 pairs that differ by presence and/or absence of just 2 alleles (Dice similarity indexes of 94.7, 92.5 and 86.1) and 2 pairs by only one allele (Dice indexes of 97 % and 97.1 %). One pair was found to have common parentage, but there is no evidence of common parentage in the pedigrees available for the remaining pairs. We have also observed some limited intra-varietal variations (data not shown) but in all cases, it was due to the presence and/or absence of a single allele of low intensity close to the cut off point set in the GeneMapper software. Most of these were addressed by an adjustment of the calling rules. There are 10 groups of potato varieties for which genotypes are identical (100 %) within the group (Fig. 1). Six of the 10 groups were tested further using 21 more microsatellite markers with no further differentiation observed (data not shown), therefore confirming the close relationship of the varieties within each group/pair. Most groups or pairs are suspected to be essentially derived one from another or represent the same variety named differently (see A, B, C, F, G, H, I, J, Fig. 1). The group of 5 varieties sharing and identical genotype (H) are probably the same blue variety but named differently (De Jong and Murphy 2003). Looking at the pedigree of the varieties composing 4 of the groups, it was observed that varieties of these groups shared common parentage or one variety was the progeny of the other one (Fig. 1 A, B, F, I). This was not observed by the European group for over 1,000 potato varieties and may introduce some doubt regarding the labelling of these particular varieties. The last two pairs (D and E) did not present any evidence of common parentage and therefore, are strongly suspected to be cases of mislabelling at one point in time.

Potato Variety Verification Testing

During the Canadian Seed Certification process, inspection of lots is carried out to verify the identity and purity of the potato varieties. If the number of rogues in a crop exceeds the tolerance level and the inspector can not confirm the identity of these morphologically, samples of leaflets or tubers are sent to the laboratory for genotyping analysis. Five different potato variety verification requests were received during the course of this study and examined using the harmonized microsatellite method. For each submission, a minimum of 2 individuals per sample of the suspected rogue were genotyped and when available, 2 normal looking individuals (normal bulk) from the same field or lot. When necessary and if available, reference tuber or plantlet samples were tested along with the samples especially when the reference genotype of the variety in question was not previously established. Of the 5 cases, there were 4 different outcomes (Fig. 1, areas labelled 1 to 5).

In one case, two seed lots with the same variety name appeared different morphologically. Leaflets from plants from each lot (MIRL11-041 & 042 and MIRL11-044 & 045) were collected and sent to the lab for genotyping (Fig. 1 area labelled 1). Reference material for the variety to be certified (plantlet MIRL11-Pot-029 and tuber MIRL11-Pot-035) were obtained from two other sources. Results are shown in Fig. 1 where the phylogenetic tree demonstrates that one of the two lots (MIRL11-044 & 045) yielded a genotype pattern different to the reference with a Dice similarity index of 65.6 %; the other lot (MIRL11-041 & 042) was identical to the reference plantlet genotype. There was no match in our database to the incorrect seed lot and, as a follow up, two other nuclear stocks (MIRL11-055 &056) were sampled and submitted to the lab to eliminate any mislabelling possibility. These were shown to be identical to the references for the variety to be certified (Fig. 1). As the variety to be certified was bred in Europe, all genotypes obtained from this analysis were shared with the European potato database for confirmation of the references and for a possible identification of the unknown. The European potato database provided confirmation of the variety for the reference genotype (UK-002) and furthermore provided an identification of the unknown genotype (UK-001). Further investigation revealed that the two varieties were bred at the same facility and registered by the same representative.

Another diagnostic request was made when two lots inspected for potato seed certification showed a high level of occurrence of plants that displayed morphological characteristics not typical to the variety being certified, thereby raising the possibility of a variety purity problem. Leaflets were sampled from individual plants morphologically representative of the variety being certified (normal bulk: MIRL10-008NB & 013NB in Fig. 1 area labelled 2) and from individuals suspected to be rogue (MIRL10-017 & 018; MIRL10-022 & 025). The samples of the variety to be certified (MIRL10-008NB & 013NB) matched with the European reference sample (UK-041). However, the rogue samples (MIRL10-017 & 018; MIRL10-022 & 025) did not match

the reference genotype profile with a Dice similarity index of only 56.4 %. Furthermore, the rogue samples did not match anything in either the European or Canadian databases. Therefore, the two inspected lots in question were not certified as they exceeded the tolerance level of rogue individuals.

In a case of a nuclear stock suspected to be mislabelled, plantlets (MIRL09-156-01 & 02) were genotyped along with reference plantlets available for the variety indicated on the label (MIRL09-Pot-001) and for the suspected mislabelled variety (MIRL09-Pot-002). Searching the genotype profile information with the European database allowed variety identity confirmation as both varieties involved were bred in Europe. Results are shown in Fig. 1 area 3 as the nuclear stock sample was confirmed to be of the right variety (Dice similarity index 100 %) and not mislabelled (Similarity index 64.9 %).

The last two cases involved two newer North American varieties for which both Canadian and European databases did not have references. However for each case, both Canadian and European databases did have a reference profile for the suspected rogue variety (respectively MB04-Pot-178 and UK-07/024 Fig. 1 area 4 & MB04-Pot-213 and UK-0842 Fig. 1 area 5). Furthermore, it was possible to obtain samples from another source believed to be of the true variety, but not confirmed genetically (MIRL11-Pot-046 Fig. 1 area 4 & MIRL11-035NB & 036NB Fig. 1 area 5). Tuber or leaflets from a minimum of 2 individuals were sampled per site (MIRL11-061 & 062; MIRL11-057 & 058; MIRL11-064 & 065 Fig. 1-4; MIRL11-038 & 039 Fig. 1 area 5) and tested. Results shown in Fig. 1 area 4, demonstrated that samples of one of the sites (MIRL11-064 & 065) did not match the other samples tested (MIRL11-058, 059, 061 & 062 with Dice similarity index of 68.2 %) or the reference tested for the variety being certified (MIRL11-Pot-046). Furthermore, the sample matched one of the varieties (MB04-Pot-178) from the Canadian database confirming the mislabelling. A similar situation was found in case #5. Figure 1 area 5 demonstrates that the samples tested (MIRL11-038 & 039) did not match the normal bulk samples (Dice similarity index 68.2 %) used as a reference (MIRL11-035NB & 036NB). Again, the samples matched one of the varieties (MB04-Pot-213) in the Canadian database confirming a mislabelling event.

Discussion

Availability of a rapid method for potato variety identification at all stages of seed production and certification is important for the industry. Traditional morphological identification is becoming more challenging and DNA markers can be used on any part of the plant therefore demand for the use of such markers is increasing (Cooke 1999). Several microsatellite based markers have been developed for the potato (Ghislain et al. 2004; Kawchuk et al. 1996; Milbourne et al. 1998). These markers have shown to be reliable, consistent and reasonably discriminative for use by several laboratories as a potato genotyping tool. For example, microsatellite markers developed for potato have been used recently to analyse the genetic diversity of potato varieties in China, Kenya and Spain (Duan et al. 2009; de Galarreta et al. 2011; Lung'aho et al. 2011). Several other groups have been using microsatellite markers for variety identification purpose in Argentina, Czech Republic, France, Korea and the United States (Cho et al. 2011; Coombs et al. 2004; Karaagac et al. 2010; Moisan-Thiery et al. 2005; Norero et al. 2002; Nováková et al. 2010). Generally for genetic diversity assessment and the production of phylogenetic trees, more than 10 markers were used while less than 10 markers were very successful in genotyping and differentiating within 17 (Coombs et al. 2004) or 286 (Moisan-Thiery et al. 2005) potato varieties. Furthermore, a method essentially developed as a potato variety verification system for varieties held in Canadian repositories allowed the identification of some 116 cultivars using only 4 SSRs (Li et al. 2008). Although all marker sets discussed are efficient in the identification of potato varieties, they also differ from one another so the genotypes produced can be compared only to those within the same study. Furthermore, as the PCR fingerprints are analysed by different gel systems (Agarose, polyacrylamide and capillary electrophoresis), scoring differs from one system to the other and therefore complicates possible exchange of genotyping data. Such exchanges would be beneficial when an unknown genotype does not match any of the genotypes within a reference collection. Standardization in the scoring of alleles was one of the goals in the establishment of a potato genetic identity kit developed by Ghislain et al. (2009). The method used a set of 24 SSR markers and discriminated 93.5 % of the 742 native potatoes. The construction of a SSR fragment size ladder for each marker aided in the scoring of bands generated by the LI-COR DNA Analyzer System. With the increasing number of varieties registered around the world and the exchange of genetic material, the construction of unified databases of variety reference genotype profiles is an invaluable asset for stakeholders. Simple and accessible methods with established allele calling rules are a requirement to minimise inter-laboratory variations which is mandatory for the establishment of such databases (Jones et al. 2008).

Transfer and Harmonization of the European Microsatellite Marker Method

The microsatellite method by Reid et al. (2009) was sufficiently detailed to allow a seamless transfer to our facility. The use of the same capillary based system for the detection

of microsatellite PCR products also facilitated the transfer. The method was rapidly setup using a set of standard cultivars common to both groups and reference alleles were defined as specific peaks produced by a marker in a specific variety. Reference alleles were previously assigned a letter code eliminating the need to correlate allele calling according to number of base pairs which generally varies between laboratories (Table 2). However, the tetraploidicity of the potato genome generated 4 alleles per marker and competition between PCR products does complicate callings for some of the alleles. In order to address this, some calling rules relative to call cutoff level were set or modified to match our allele calling to those of Reid et al. (2011). After these adjustments, some differences still existed between the two laboratories relative to the reference genotype of 4 varieties (Fig. 1). However, there are no significant impacts from this, as the same variety genotypes still co-segregated and recording of the variation within the database can alert the user to the need to account for differences for these particular varieties, depending on the visualization system used or the laboratories generating the genotype(s). The local database is therefore even more powerful as it informs its user of possible inter-laboratory variation.

Analysis of the 224 Reference Potato Varieties

The method differentiated most varieties in our collection. A total of 89 % of the CFIA collection presented a unique genotype. When both AFLP and SSR methods were used in some cases of potato variety verification (data not shown), results were in agreement. However an advantage of the microsatellite method is that genotypes are composed of fewer bands per reaction and the patterns are more consistent from one sample to another than AFLP fingerprints, it is, therefore, easier to identify unique genotypes from those common to pairs or group of varieties. Since attempts to further differentiate pairs or groups of identical genotypes using more microsatellites did not reveal further differentiation, the limitation of the current method is not in the choice or number of markers used but probably linked to the genotyping of essentially derived material, or of the same line sold under different names. This shows the reliability and robustness of the marker system for the purpose of varietal verification. Availability of the same line under a different name or different lines under the same name, have occurred with older varieties before current registration systems were available (De Jong and Murphy 2003). This could have been the case for garden variety grade potatoes (for example the blue potatoes) which were excluded from official registration until only very recently. Information on pedigree may be limited for some of the different varieties displaying identical genotypes, but names that have suffixes such as red, or purple are a good indication of cases of mutation or clonal variation. Cases of identical genotypes that cannot be explained by similar pedigree or other reasons explained earlier are most likely due to mislabelling, which is always possible for potato, as the germplasm maintenance requires more human handling than most crops.

Potato Variety Verification Testing

Trueness to type is required for seed potato tubers to be certified by the Canadian government. As the seed class level increases, the percentage of tolerated rogue tuber decreases. Therefore, the percentage of rogue tubers in a lot cannot exceed the percentage allowed for a particular class. The potato samples genotyped for varietal verification in this study were taken during routine inspection of seed lots (4 submissions) and of nuclear stock (1 submission). For all 5 cases, morphological considerations led the inspector to believe that the lot or stock being inspected contained all or high levels of rogues and it was therefore ineligible for Seed Certification. Molecular genotyping was able to provide confirmation of the morphological discrepancies observed during the inspection.

The genotyping results confirmed the trueness to type of the nuclear stock variety (Fig. 1 area #3). Potato plantlets do not always display enough differences in the morphological characteristics for varietal identification therefore genotyping is an invaluable addition for the maintenance of germplasm. Mislabelling of nuclear stock has a significant impact for the producers as all seed potatoes derived from mislabelled germplasm may not be certified. If the seed potatoes cannot be sold at the desired certification level, there is a significant loss to the producer. The 4 other cases (Fig. 1 area 1, 2, 4 and 5) were samples taken from seed potato production fields to be certified. All were suspected to be rogue and confirmed not to be of the correct variety. For the cases identified in areas 1, 4 and 5, it was possible to identify the rogue variety by comparison with the reference genotype. Occasionally, different seed lots and germplasm of the varieties in question were genotyped to help determine the origin of the mix-up. Corroborative evidence from the European genotype database was proven to be invaluable especially when European-bred varieties were involved. The combination of the genotypes from this work with the European genotypes allowed searching for reference genotypes of over 1,200 potato varieties. Confirmation of the presence of rogue seed potato varieties directly impacted the Canadian Seed Potato Certification system by protecting the integrity of production. Without these detections, rogue and incorrect varieties would have been distributed throughout the system, ultimately resulting in greater financial losses to producers.

Conclusion

The SSR markers established by the European community laboratories were successfully employed in the Canadian setting as most genotypes generated were identical. The method successfully differentiated 200 potato reference DNAs of the Canadian collection with the exception of 10 groups that were most likely clonal variants. The SSR markers were applied to address potato variety verification requests from CFIA inspectors and enable CFIA to successfully fulfill its mandate to support potato stakeholders.

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