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Identification of Canadian durum wheat varieties using a single PCR

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Abstract Accurate and reliable means of variety identification are necessary to assess purity of seed supplies, to support claims relating to plant breeders' rights and, in Canada, to provide quality assurances in the grain handling system. A single, multiplexed set of seven simple-sequence-repeat (SSR) markers was found to uniquely identify all 18 durum wheat varieties that have been developed in Canada and are currently, or were formerly, registered for commercial production. Significant features of this multiplexed set include an allele that is specific, within Canadian durum varieties, to those having high gluten strength, and redundancy that was included in an effort to increase the capacity to accommodate future varieties. In combination with a reasonably rapid individual-kernel DNA extraction protocol and automated allele calling, this marker system offers a higher resolution alternative to complement established protein-based variety identification methods.

Introduction

Canada is one of the largest producers of durum wheat (*Triticum turgidum* L. var *durum*) and is the largest exporter of durum to world markets. The quality and consistency of Canadian durum is maintained by strict grading standards and strict control of varieties. Durum may be segregated from other classes of wheat grown in Canada based upon visually distinguishable kernel characteristics. However, in recent years there has been an increasing demand for a finer scale of segregation based on end-use quality characteristics. For example, the variety AC Navigator has high gluten strength (Clarke et al. 2000a), a characteristic that contributes to the "al dente" quality of pasta preferred by some consumers and, although it is not visually distinguishable from conventional-strength durum, it is being marketed on a variety-specific basis. In addition to providing assurances in segregated handling, a robust means of variety identification is also required for the assessment of seed purity and the protection of plant breeders' rights.

Electrophoresis (Zillman and Bushuk 1979; Tkachuk and Mellish 1980) and reversed-phase high-performance liquid chromatography (RP-HPLC) (Marchylo et al. 1988; Scanlon et al. 1989) of seed storage proteins are well-established and often effective methods for identification of cereal varieties. But the ability of such biochemical analyses to distinguish among varieties, including those of durum, is not absolute (Mellish, unpublished data). Alternatively, DNA-based methods offer the potential of much greater resolving power because the number of markers possible is essentially limitless. DNA markers are also attractive because they do not vary among tissues or developmental stages of a plant, and they are not affected by other environmental factors.

Although DNA restriction fragment length polymorphisms (RFLPs) have been proposed for durum variety identification (Pagnotta et al. 1996), markers that are based upon the polymerase chain reaction (PCR) have received more attention (Pasqualone et al. 1999; 2000; Dograr et al. 2000; Soleimani et al. 2002). PCR-based markers generally require very small amounts of DNA, permitting relatively rapid analysis of single kernels. Soleimani et al. (2002) have described an identification scheme for Canadian durum varieties using sequence-tagged-site (STS) markers. Their system involves seven STS markers, each amplified under different PCR conditions; two of the markers also require digestion with differing restriction enzymes. Clearly, that approach is not readily adaptable to high throughput.

The aim of this research was to establish a more rapid and efficient variety identification system for Canadian durum wheat. This goal was achieved through the use of a single set of multiplexed simple-sequence-repeat (SSR or microsatellite) markers.

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Materials and methods

Seed material

Sampling included all past and present durum varieties developed and registered in Canada (Table 1). Breeder seed was used for the 14 varieties that were registered in Canada at the outset of this research (interim registration for AC Pathfinder has since lapsed). Seeds of four varieties that were developed in Canada but are no longer registered for production and sale were obtained from historical collections held by the Canadian Grain Commission.

DNA extraction

DNA was extracted from single kernels using a procedure adapted from McDonald et al. (1994). Dry kernels were crushed with needle-nosed pliers as they were individually placed into 2-μl microcentrifuge tubes (Sarstedt) containing two 0.25-in ceramic spheres (Qbiogen). Up to 48 individual kernels were then pulverized simultaneously for 1 min at 30 Hz in a Type MM 300 vibration mill (Retsch) equipped with a 2×24 adapter set (Qiagen), followed by the addition of 1,250 μl of extraction buffer [200 mM Tris-HCL (pH 7.5), 288 mM NaCl, 25 mM EDTA and 0.5% SDS], agitation for an additional 30 s at 30 Hz and centrifugation for 5 min at 6,200 rpm (6,189 *g*) in a 4K15 centrifuge (Sigma) fitted with an 09100 rotor (Qiagen). A portion of the supernatant (750 μl) was combined with an equal volume of isopropanol, and the precipitated DNA was pelleted by centrifugation for 2 min at 5,300 rpm (4,522 *g*). Pellets were air dried for 20 min and resuspended in 200 μl autoclaved, nanopure water.

Source of SSR markers

SSR markers were selected from those previously characterized in 64 durum wheat selections, landraces and varieties (Eujayl et al. 2002). They included EST-derived SSRs (loci designated *DuPw*, primers designated DuPw) developed by Eujayl et al. (2002), and SSRs from total genomic DNA (loci designated *Xgwm*, primers designated WMS) previously reported by Röder et al. (1998). Only markers found by Eujayl et al. (2002) to have more than two alleles and a diversity index greater than 0.5 were considered. Twenty-one markers satisfied these criteria.

PCR and fragment analysis

PCR primer sequences were as listed by Eujayl et al. (2002) or Röder et al. (1998) except that each forward (5′) primer was 5′ tailed with the M13 sequence 5′-CACGACGTTGTAAAACGAC-3′. The same amplification conditions were applied to all markers. Reaction mixtures (10 μl total volume) contained 1 μl DNA solution, 0.05 μM each SSR-specific primer, 0.05 μM IRDye700-labeled M13-forward (-29) primer (Li-Cor) that matched the sequence of the M13 tail, 0.20 mM each dNTP, 1.5 mM $MgCl_2$, 0.5 U Ampli*Taq* DNA polymerase (Applied Biosystems) and 1× of the supplied reaction buffer. PCR was performed in a PTC200 thermal cycler (MJ Research). An initial cycle (94°, 3 min; 58°, 1 min; 72°, 1 min) was followed by 30 cycles (94°, 30 s; 58°, 30 s; 72°, 30 s). A final extension was performed at 72° for 5 min.

Electrophoresis and visualization of SSR alleles was carried out using an IR^2 Gene ReadIR 4200 DNA Analyzer (Li-Cor). Gels, 25-cm long and 0.25-mm thick, contained 6% Long Ranger gel solution (BMA) and 7 M urea in 1×TBE. Following addition of 5 μl stop/loading buffer [95% formamide, 0.1% bromophenol blue, 20 mM EDTA (pH 8)] and denaturation at 95° for 5 min, 0.8 μl of reaction mixture was loaded into wells formed by a 64-well, square-toothed comb and subjected to electrophoresis under constant power (40 W) at 45° for 1 to 1.5 h. An IRDye700-labelled 50–350 bp concentrated sizing standard (Li-Cor) was included (0.2 μl) at approximately 16-lane intervals to facilitate fragment size determination and automated allele calling using Saga Generation 2 software (Li-Cor).

Table 1 Durum wheat varieties developed and registered for production in Canada

Variety	Year registered	Parentage
Pelissier	1936	Selection from Algerian stock
Stewart 63	1963[a]	St464/8*Stewart
Hercules	1969	RL3097/RL3304//Stewart/RL3380
Wascana	1971	Lacota*2/Pelissier
Wakooma	1973	Lacota*2/Pelissier
Macoun	1974[a]	RL3607/DT182[c]
Coulter	1977[a]	DT188/DT 224//DT182[d]
Medora	1982	Ward/Macoun
Arcola	1983[a]	Wascana/Hercules
Kyle	1984	Wakooma/DT322//Wakooma/DT320
Sceptre	1985	68111/Ward//Coulter
Plenty	1990	Vic/Wascana//Hercules/DT310
AC Melita	1995	Medora/Lloyd
AC Morse	1996	DT430/DT437//DT367/Medora
AC Avonlea	1997	DT379/DT367//DT367/Medora
AC Pathfinder	1998[b]	Westbred 881/DT367
AC Navigator	1998	Kyle/Westbred 881
Napoleon	2002	Vic/DT384//DT471

[a] Deregistered
[b] Interim registration expired
[c] RL3607 and DT182 are sister selections of Hercules
[d] DT182 and DT188 are sister selections of Hercules

Marker screening and development of a multiplex assay

Initially, the amplification quality and level of polymorphism was assessed for each marker using a screening panel comprising four kernels of each of the 14 registered durum varieties. Allele frequencies were estimated based upon the 56 seeds in this panel and the diversity index (DI) of each marker locus was calculated as $DI=1-\sum p_i^2$, where p is the frequency of the ith allele at the locus (Weir 1990). A set of seven markers that had non-coincident alleles and provided sufficient information to discriminate among all 14 varieties was selected. Multiplex PCR of these seven markers in a single reaction was carried out under the same conditions as PCR of individual loci, except that some primer concentrations were adjusted to allow a more even distribution of product intensities among the markers. Multiplex PCR was performed on 30 kernels of each of the 14 registered varieties and on 12 kernels of each of four formerly registered varieties.

Results

All of the 21 primer pairs considered directed clean amplification of products that were within expected size ranges, taking into account an additional 19 bp corresponding to the M13-tail. For one marker (*DuPw227*), a single monomorphic product was observed in the initial screening panel (4 kernels × 14 varieties). For several others (*DuPw023*, *DuPw043*, *DuPw205*, *DuPw238*, *Xgwm526*) two fragments were amplified, perhaps corresponding to separate loci in the A and B genomes of durum. In these cases, at least one of the loci was polymorphic in the screening panel. Loci that were polymorphic in the screening panel are listed in Table 2.

Table 2 Polymorphism of simple-sequence-repeat markers observed in four kernels from each of 14 Canadian durum varieties. Entries in *bold* indicate markers that were included in a multiplexed variety identification marker set. *DI* Diversity index

Locus[a]	Number of alleles[b]	Allele sizes (bp)	DI
DuPw004	**3**	**213, 215, 218**	**0.66**
DuPw023b	2	251, null	0.24
DuPw038	2	205, 217	0.24
DuPw043a	2	248, null	0.50
DuPw043b	3	251, 254, 260	0.22
DuPw115	**2**	**202, 208**	**0.48**
DuPw124	2	177, 183	0.22
DuPw167	**3**	**244, 258, 260**	**0.47**
DuPw205a	**3**	**183, 188, 193**	**0.50**
DuPw216	2	195, 204	0.19
DuPw217	**3**	**229, 232, 241**	**0.57**
DuPw238a	2	228, 234	0.16
DuPw254	2	173, 175	0.13
DuPw398	2	202, 214	0.34
Xgwm099	**4**	**129, 137, 141, 151**	**0.70**
Xgwm164	2	142, 146	0.13
Xgwm169	2	205, 209	0.13
Xgwm193	3	186, 188, 198	0.41
Xgwm304	2	215, 223	0.04
Xgwm526a	**3**	**157, 161, 163**	**0.60**
Xgwm526b	**2**	**165, 167**	**0.34**
Xgwm544	3	198, 200, 206, 216	0.67

[a] Where two loci were amplified using a single primer pair, the loci were designated as *a* and *b*, with locus *a* having the lower molecular weight

[b] Additional alleles were observed upon subsequent examination of a multiplexed variety identification marker set in a larger number of kernels and varieties. These included *DuPw167-264* and *266*, *Xgwm526b-171* and *Xgwm099-133*. The allele *Xgwm099-133* presented a complex banding pattern with additional primary bands at 149 bp and 155 bp

Seven primer pairs that directed amplification of a total of eight polymorphic loci were successfully combined into a single multiplex reaction that uniquely identified each of the registered Canadian durum varieties (Fig. 1). Primer concentrations in the multiplex PCR either remained at 0.05 μM as in single-locus PCR (*DuPw004*, *DuPw205*, *DuPw217*, *Xgwm526*) or were increased to 0.075 μM (*DuPw115*, *DuPw167*, *Xgwm099*). Observed allele size ranges at these loci did not overlap. However, owing to the proximity of their alleles, *DuPw526a* and *DuPw526b* were treated as a single locus with each individual kernel scored as a heterozygote. Automated allele calling was generally accurate, but visual verification of the results was required to ensure that stutter bands or other artifacts were not occasionally miscalled as alleles. Also, allele *DuPw167-244* was very strongly amplified and at times interfered with the automated scoring of *DuPw217*, most notably when allele *DuPw217-241* was present as in Pelissier and AC Melita.

The multiplexed marker combination generally included the loci that had the highest diversity indices in the initial screening panel (Table 2). Although *DuPw167* had lower diversity than the similarly sized *DuPw043a/b*, it was chosen for inclusion in the multiplexed set because it allowed discrimination between AC Melita and Plenty and between Morse and Medora biotype B. The multiplex reaction also functioned well if *DuPw043a/b* were substituted for *DuPw167*. In a test of the capability of the multiplexed SSR set to discriminate among additional varieties, four Canadian durum varieties that are no longer registered (Stewart 63, Macoun, Coulter and Arcola) were also uniquely identified. A complete listing of SSR genotypes of all durum varieties developed in Canada is presented in Table 3.

Polymorphism within varieties was revealed by the multiplexed SSRs in three cases. Medora had two biotypes that differed at *DuPw115*, while two Arcola biotypes differed at *DuPw004*. Pelissier was the most heterogeneous, displaying two alleles at each of three marker loci (*DuPw167*, *DuPw205* and *Xgwm099*). Assuming that all combinations of alleles were possible among the marker loci, a total of eight Pelissier biotypes were predicted. In the 30 Pelissier kernels initially genotyped, only five of these expected biotypes were observed, with biotype G predominating. The total number of kernels of Pelissier breeder seed examined was then increased to 150 and ultimately seven of the eight expected biotypes were recorded (Fig. 2).

Discussion

Unique identification of each durum wheat variety registered for commercial production in Canada was accomplished using a single multiplexed set of SSR markers. Table 3 may be used as an identification key. It is arranged such that the markers are listed in the order the data are generated on a gel (i.e., lowest to highest molecular weight) and the order of varieties is determined by the alleles present at successive loci. As more data are generated during a run, one may move from left to right across the table, narrowing the pool of candidate varieties, ultimately to one. However, in a high-throughput system, one would likely forgo manual determination of varieties and simply export data collected by automated allele-calling software directly to a routine that would match observed genotypes to those in a variety database.

A convenient feature of the proposed identification scheme is that AC Pathfinder and AC Navigator, the two Canadian varieties having high gluten strength (Clarke et al. 2000a, 2000b) are also the only varieties to possess the allele *Xgwm099-151*. This association is likely a reflection of common parentage (AC Pathfinder and AC Navigator both derive from crosses involving the American variety Westbred 881, Table 1) rather than linkage of *Xgwm099* to a gene or region affecting gluten strength. Nonetheless, since *Xgwm099-151* may be detected early in electrophoresis, it provides a means for rapid recognition of the current extra-strong varieties.

In some cases, markers were found to be polymorphic within varieties. This is not viewed as a problem for variety identification, since all biotypes that were found or are expected to occur may be assigned unambiguously to a specific variety. Pelissier is the most heterogeneous

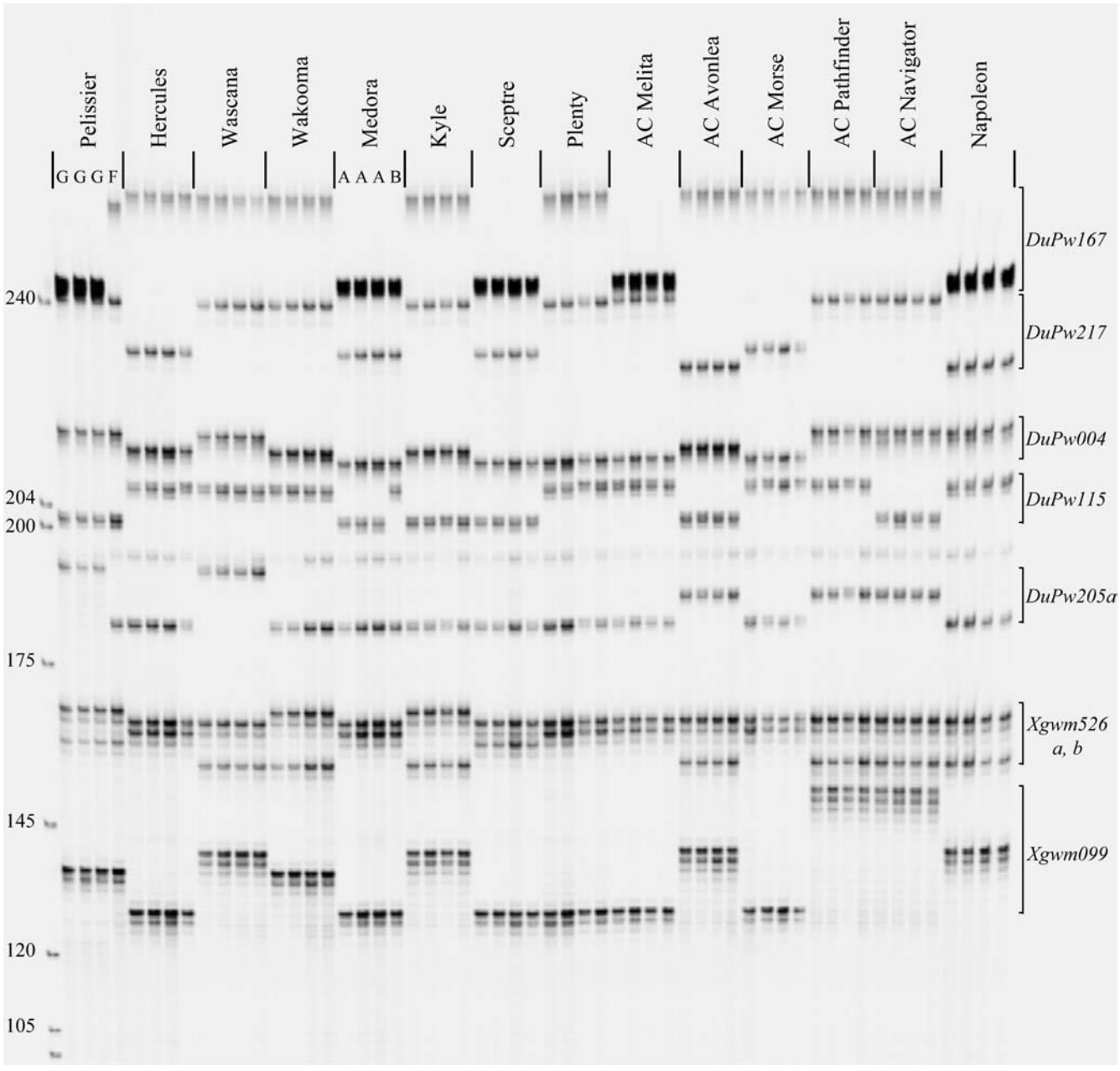

Fig. 1 Simple-sequence-repeat (SSR) profiles of fourteen Canadian durum wheat varieties. A total of eight polymorphic SSR markers were amplified in a single, multiplex PCR containing seven primer pairs. Each *lane* represents a single durum kernel, with four kernels shown per variety. Where polymorphism within a variety is evident, *letters above the lanes* correspond to biotype designations (Table 3). The *leftmost lane* is a 50–350 bp concentrated sizing standard (Li-Cor) with fragment sizes (bp) as indicated

of the varieties. It is an old variety, originally from Algeria and introduced into the United States in 1900. The stock grown in Canada derives from a selection made at the University of Saskatchewan in 1921 (Knott 1995). Whether the variety that is still grown to some extent today has remained unchanged since the original selection was made cannot be determined from these data. However, in addition to the Pelissier breeder seed analyzed, which derived from two different lots of seed, a modern producer's sample and an older historical sample (of unknown origin) were also examined (data not presented). In all cases the same polymorphisms were found, indicating consistency of the variety, at least in recent times.

Not all markers in this set are essential for complete discrimination among the varieties; either *DuPw205* or *Xgwm099* may be excluded without affecting the outcome. However, the inclusion of more markers than are currently necessary is intended to provide additional capacity to accommodate the introduction of new varieties in the future. Given the numbers of alleles observed at these eight marker loci, it should be possible to discriminate 12,150 unique genotypes. However, the

Table 3 Identification of Canadian durum varieties based on a single multiplex set of simple-sequence-repeat markers. Where polymorphism within a variety was observed, biotypes were each given a single letter designation

Variety	Observed biotype frequency	Allele sizes (bp)							
		Xgwm099	*Xgwm526a*	*Xgwm526b*	*DuPw205*	*DuPw115*	*DuPw004*	*DuPw217*	*DuPw167*
Sceptre	-	129	161	165	183	202	213	232	244
Coulter	-	129	161	165	183	202	218	232	266
Medora	A 22/30	129	163	165	183	202	213	232	244
	B 8/30	129	163	165	183	208	213	232	244
AC Morse	-	129	163	165	183	208	213	232	260
AC Melita	-	129	163	165	183	208	213	241	244
Plenty	-	129	163	165	183	208	213	241	260
Hercules	-	129	163	165	183	208	215	232	260
Macoun	-	129	163	165	183	208	218	232	260
Pelissier	A 1/150	133[b]	161	167	183	202	218	241	244
	B 0/150[a]	133	161	167	183	202	218	241	258
	C 14/150	133	161	167	193	202	218	241	244
	D 7/150	133	161	167	193	202	218	241	258
Wakooma	-	137	157	167	183	208	215	241	260
Pelissier	E 1/150	137	161	167	183	202	218	241	244
	F 7/150	137	161	167	183	202	218	241	258
	G 105/150	137	161	167	193	202	218	241	244
	H 15/150	137	161	167	193	202	218	241	258
Napoleon	-	141	157	165	183	208	218	229	244
AC Avonlea	-	141	157	165	188	202	215	229	260
Wascana	-	141	157	165	193	208	218	241	260
Kyle	-	141	157	167	183	202	215	241	260
Arcola	A 6/12	141	163	165	183	208	215	241	260
	B 6/12	141	163	165	183	208	218	241	260
Stewart 63	-	141	163	171	183	202	218	241	264
AC Navigator	-	151	157	165	188	202	218	241	260
AC Pathfinder	-	151	157	165	188	208	218	241	260

[a] Biotype B of Pelissier was not observed but was predicted to occur at a low frequency
[b] Allele *Xgwm099-133* presented a complex banding pattern with additional primary bands at 149 and 155 bp

actual probability that two varieties will be the same at all eight loci will depend upon the allele frequencies in the breeding population and upon the relatedness of the varieties in question. In reality, this probability is likely much greater than the total possible number of unique genotypes would suggest. Nonetheless, it is of some reassurance that the four formerly registered varieties that were genotyped after the marker combination was established were found to be distinct, even though some are quite closely related to currently registered varieties.

It remains to be determined whether this multiplexed set of SSR markers will be useful for varietal identification in other groups of varieties such as durums from other sources or even wheats of other classes. Eujayl et al. (2002) identified nine SSRs that they considered to be the best combination for genotyping. The current set of seven SSRs was selected from the same marker pool but includes only three of the same SSRs. This difference may be due in part to the selection of the multiplexed markers being governed somewhat by constraints in addition to allele frequencies, such as a requirement of noncoincident alleles. However, it is noteworthy that the marker that Eujayl et al. (2002) found to be the most informative in 64 durum accessions, land races and varieties (*Xgwm169*, 14 alleles, DI=0.96) was among the least variable in Canadian durums (2 alleles, DI=0.13). Clearly, the level of polymorphism of specific markers in different gene pools is unpredictable.

The protocol described here, using a Li-Cor automated DNA analyzer, allows the amplification products of 60 kernels to be evaluated in one loading of a single gel. In addition, throughput may be doubled by simultaneously loading reactions having IRDye800-labeled, M13-forward primer rather than its IRDye700-labeled counterpart. Since each gel may be used three times, it should be possible to analyze 360 kernels on a single gel over a period of about 6 h. Performing PCR to obtain this number of products is fairly rapid. Setup is straightforward since a single common reaction mixture, containing either IRDye700-labeled or IRDye800-labeled, M13-forward primer, may be distributed throughout one or more 96-well plates. PCR can be completed within 1.5 h in a conventional thermal cycler.

The current rate-limiting step is DNA extraction. In applications where leaf tissue can be used, high-throughput, 96-well-plate-format plant DNA extraction protocols (e.g., Dilworth and Frey 2000; Paris and Carter 2000) or 96-well-format commercial kits may be appropriate. However, in the context of a grain handling system, varietal identification is often required in a short time frame, precluding germination and growth of material for DNA extraction and making extraction from individual kernels a necessity. Durum kernels are pulverized very

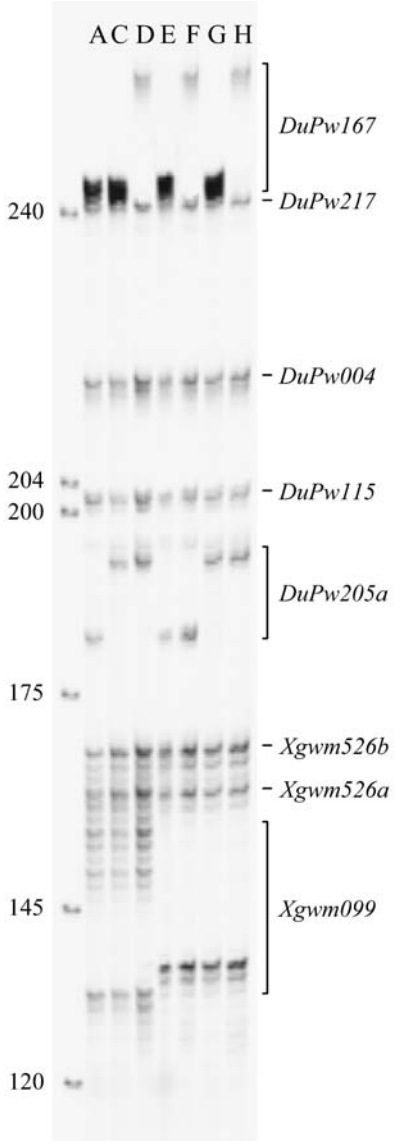

Fig. 2 SSR polymorphism observed within Pelissier durum wheat. With three SSR markers (*DuPw167*, *DuPw205*, *Xgwm099*) polymorphic within Pelissier, eight possible SSR profiles were predicted. Examples are shown for each of the seven profiles observed among 150 individual kernels of Pelissier breeder seed examined. *Letters above the lanes* correspond to biotype designations (Table 3). The *leftmost lane* is a 50–350 bp concentrated sizing standard (Li-Cor) with fragment sizes (bp) as indicated

effectively using the procedure described here, and the subsequent extraction of DNA is reasonably rapid since few pipetting steps and only one transfer step are required. Experience indicates that 48 individual kernels (the capacity of the vibration mill adapter set) can be processed in about 1 h, not including drying or resuspension time. DNA can also be extracted from other sources, such as pasta or semolina (Pasqualone et al. 1999; Alary et al. 2002), but identification of the varietal origin of such products will only be possible if they derive from a single variety. Varietal composition of seed or grain samples may be ascertained through the analysis of multiple single kernels.

Until such time as technological advances permit routine and rapid interrogation of DNA sequence polymorphisms directly, without prior DNA amplification, the application of the multiplexed SSR marker set described here may be among the least expensive and quickest means available for DNA-based identification of durum wheat varieties. Admittedly, owing to the amount of hands-on time required, the high costs of reagents such as *Taq* polymerase and licensing requirements related to PCR, it will not be cost competitive with, nor is it expected to supplant well-established, but lower resolution methods such as electrophoresis of seed storage proteins. Rather, it is offered as an additional tool in a varietal identification toolbox, intended for use when the benefit of absolute resolution of varieties is sufficient to justify the additional cost.

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