JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Detection of Soft Wheat in Semolina and Durum Wheat Bread by Analysis of DNA Microsatellites

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The aim of this work was to evaluate the analysis of DNA microsatellites for the detection of soft wheat (*Triticum aestivum* L.) in semolina and durum wheat bread (prepared from *Triticum turgidum* L. var. *durum*). The results enabled selection of an efficient D-genome-specific repetitive DNA sequence to detect common wheat in semolina and breads by qualitative PCR with a threshold of 3 and 5%, respectively, lowered to 2.5% by real-time PCR. This is of major importance for checking during production of some typical products recently awarded the European Protected Designation of Origin (PDO) mark such as Altamura bread, which should not contain soft wheat flour. The feasibility of quantification of common wheat adulteration in semolina using real-time PCR was also demonstrated.

KEYWORDS: Durum wheat; bread; DNA microsatellites; soft wheat detection; polymerase chain reaction

INTRODUCTION

The detection of common wheat (*Triticum aestivum* L.) in durum wheat (*Triticum turgidum* L. var. *durum*) semolina has always been the object of interest and stimulated the development of numerous analytical methods, generally aimed at searching and identifying specific common wheat protein fractions. Electrophoretic methods based on such a principle were recommended as early as in 1960s by Resmini (*1*) and Garcia-Faure et al. (*2*), followed by the immunoenzymatic versions set up by Cantagalli (*3*) and by Stevenson (*4*). Regarding the analysis of the end-products such as pasta, the evolution of the drying process, with the increase of adopted temperatures, affected these early methods because of significant protein denaturation. As a consequence, some commercial immunodetection assays specific for pasta samples, such as Pastascan (R-Biopharm Rhône Ltd), have to incorporate a test to determine the degree of heat treatment of the sample, to allow the selection of the most appropriate heat-treated pasta standards to be used in the assay.

More recently, a new generation of methods which employ DNA screening for sequences localized in the D-genome, characteristic for common wheat, has become available (*5*−*8*). Owing to the relative stability of DNA molecules at the temperatures applied for pasta drying, the development of these systems has been mainly directed to the detection of common wheat in pasta. In fact, the manufacture of dried pasta from hexaploid wheat or from its mixtures with durum wheat, without adequate labeling, is considered to be an adulteration. Only a 3% presence of *T. aestivum* is allowed, accounting for accidental contamination during the agricultural process (*9*).

On the other hand, also some baked products like the typical breads from Southern Italy, namely those from the towns of Altamura and Matera, have to be prepared by using durum wheat semolina (*10*, *11*). The typicality of these products, in fact, is related to the exclusive use of semolina derived from certain cultivars of durum wheat (*12*), grown in well defined and restricted geographic areas, and only the fulfillment of these requirements allows the European marks of Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) (*13*). Hence, the use of common wheat flour in the preparation of PDO or PGI durum wheat breads represents an adulteration.

In this framework, providing an effective method of detection of *T. aestivum* would meet and support consumers' expectations of an original product. However, during processing, bread undergoes much more drastic thermal conditions than pasta; moreover, it is subjected to the leavening process with consequent exposure of DNA to yeast nucleases (*14*).

Microsatellites are sequences of repeated DNA that can be analyzed by means of a single PCR reaction, providing short-sized amplicons (*15*, *16*). The small size of generated amplicons (around 200 base pairs) is crucial if analyzing food samples subjected, during their production process, to high temperature

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10.1021/jf063383e CCC: $37.00
Published on Web 03/30/2007

and/or strong mechanical treatments (*17*, *18*). Previous studies carried out on olive oil DNA demonstrated that the detection of short targeted sequences, such as microsatellites, is still possible despite serious DNA degradation (*19*).

Among the numerous types of DNA markers commonly used nowadays in many research fields (*20*, *21*), microsatellite sequences are clearly superior to the others because of the relative simplicity of their application and reproducibility of the results (*22*).

The aim of this work was then to evaluate the effectiveness of the analysis of some selected D-genome specific wheat microsatellites in developing a microsatellite-based protocol for the detection of common wheat in semolina and durum wheat bread.

MATERIALS AND METHODS

Samples. Grains from pure durum wheat cultivars Appulo, Arcangelo, Duilio, Norba, and Simeto, grown at the experimental field of Genetics and Breeding Section, DIBCA Dept., Bari University (Bari, Italy), were separately milled to semolina by means of a MLU202 mill (Buhler, Uzwil, Switzerland). Flour made of pure common wheat cultivars Pandas and Centauro, from the same experimental field, were prepared by grinding grains by a Labormill 4RB (Bona, Monza, Italy). Bread-making trials were carried out at a local bakery (Digesù, Bari, Italy) from each semolina and flour according to the following protocol: 500 g flour/semolina, 20 g compress yeast, 12 g sodium chloride, and about 600 mL water were mechanically kneaded for 15 min. After manual portioning and shaping, the dough was left to rise at 28−30 °C for 1 h and then manually kneaded for few minutes, shaped, and again left to rise for 1 h. Then a portion was dry-frozen and powdered to be analyzed for examination of DNA degradation level. Subsequent baking was carried out at 250 °C for 45 min. After being baked and cooled, the crumb was dry-frozen and powdered in a mortar for molecular analyses. Separate baking trials were conducted after accurate cleaning of all items. The same bread-making procedure was applied for Appulo semolina and Pandas flour, mixed in the ratios 60:40, 70:30, 80:20, 90:10, 95:5, 97:3, and 97.5:2.5. One commercial common wheat flour and one commercial durum wheat semolina (unknown mixtures of various wheat cultivars), together with the corresponding breads, were kindly provided by Digesù bakery (Bari, Italy). A commercial sample of soft wheat pasta dried at high temperature was purchased in a supermarket in Holland.

DNA Extraction. A 20 mg amount of flour or semolina and 40 mg amount of ground dry-frozen crumb, or dry-frozen leavened dough, or pasta served to extract DNA using Gene Elute Plant kit (Sigma, St. Louis, MO), with the following modifications to the manufacturer's instructions: in the first step of the protocol 500 μL instead of 350 μL of lysis solution A, and 60 μL instead of 50 μL of solution B, were added; in the second step 170 μL instead of 130 μL of precipitation solution was used; in the third step 900 μL instead of 700 μL of binding solution was added, with a final elution of the extracted DNA in 100 μL 1× TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Microsatellite Primer Sequences. A total number of 10 primer pairs was used: BARC 006, BARC008 (*23*); GDM111 (*24*); GWM52, GWM174, GWM186, GWM194 (*25*); WMC41, WMC245, WMC167 (*23*). The sequences, annealing temperatures, and mapping information are reported in the corresponding references. Primers were synthesized by Sigma Genosys (St. Louis, MO).

Qualitative PCR: Amplification and Detection of Microsatellite Markers. Amplification reactions were performed in a I-Cycler programmable thermal cycler (Bio-Rad Laboratories, Hercules, CA) in a reaction mix with the following composition: 30 ng di DNA, 1× PCR buffer, dNTP 0.25 mM, primer *forward* and *reverse* 2.5 μM each, RED*Taq* DNA polymerase (Sigma, St. Louis, MO) 1U, in a volume of 25 μL. The amplification conditions: 5 min at 95 °C; 35 cycles composed of 1 min at 95 °C, 1 min at the appropriate annealing temperature (*23*−*25*) and 2 min at 72 °C; final elongation at 72 °C for 10 min. The amplification products were separated by electrophoresis on 2.5% agarose gels in 1× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA), stained by ethidium bromide and visualized under UV light. Fragment sizes were quantified by comparison to 100-base pair molecular-size marker (Bio-Rad Laboratories, Hercules, CA) and to 0.5−10 kb marker (Sigma, St. Louis, MO).

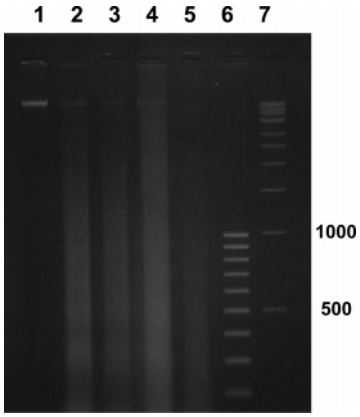

Figure 1. Agarose gel (0.8%) loaded, from left to right, with (1) λDNA, followed by DNA extracted from (2) semolina, (3) pasta, (4) leavened dough, and (5) bread, (6) molecular weight marker 100 bp, (7) molecular weight marker 0.5–10 kb.

Real-Time PCR: SYBR Green Detection. Reactions for the real-time PCR using SYBR Green detection were performed in a MX 3000 P (Stratagene, San Diego, CA) station. The amplification mix, put in 0.2 mL MicroAmp Optical reaction tubes (Stratagene, San Diego, CA), consisted of 12.5 μL Brilliant SYBR Green Q-PCR Master Mix (Stratagene, San Diego, CA), 160 nM forward and reverse primers of microsatellite GDM111 (*24*), 50 ng DNA template, and water to 25 μL. All PCR samples and negative controls (no template control, NTC, and 100% durum wheat control) were prepared in triplicate. The PCR mixture was denatured at 95 °C for 10 min; then 40 amplification cycles were carried out at 95 °C for 30 s, followed by 58 °C for 30 s, and extension at 72 °C for 30 s. A final step at 76 °C for 15 s was added at each cycle for fluorescence detection. Following the final PCR cycle, the reactions were heat-denatured from 58 °C to 95 °C for 30 min, for melting curve analysis.

RESULTS AND DISCUSSION

Figure 1 shows the extent of the degradation of DNA extracted from the samples examined. An increasing level of fragmentation was found to affect DNA, comparing semolina to pasta, leavened dough, and finally bread. Hence, DNA degradation was particularly evident in bread, with the majority of DNA fragments being below 1000 base pairs. These results agreed with those from other studies (*6*, *26*). Extraction of DNA was carried out by using a commercial extraction kit for plant DNA, but some adjustments were set up to take into account the greater water binding capacity of our samples, rich in starch and proteins, with respect to the fine particles of leaves. In particular, increased volumes of the extracting solutions provided by the manufacturer were used.

After DNA extraction from all the flour and breads, PCR amplification was carried out with a primer set designed for a microsatellite sequence (GWM186) known to be localized in A genome, that is present both in common wheat (hexaploid, $2n = 42$, AABBDD) and in durum wheat (tetraploid, $2n = 28$, AABB). This first amplification had the value of a positive control, to check the ability of all DNA samples to be amplified, prior to the subsequent analyses. **Figure 2** shows the amplification profile obtained using this primer set with DNA extracted from several samples of flour and breads from both durum and common wheat. It was observed that, in spite of DNA degradation, all the samples were correctly amplified, giving

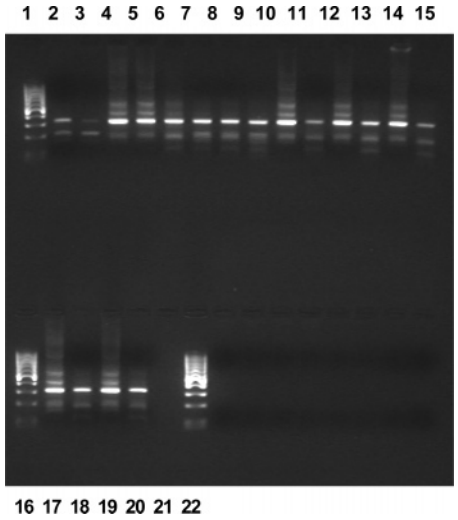

Figure 2. Amplification profile of primer GWM186. Upper part, from left to right: (1) molecular weight marker 100 bp; semolina from cv. (2) Appulo, (3) Duilio, (4) Simeto, (5) Arcangelo; bread from cv. (6) Appulo, (7) Duilio, (8) Simeto, (9) Arcangelo; (10) semolina and (11) bread from cv. Norba; (12) commercial durum wheat semolina and (13) the corresponding bread; (14) commercial soft wheat flour and (15) the corresponding bread. Bottom, from left to right: (16) marker 100 bp; (17) flour and (18) bread from cv. Pandas; (19) flour and (20) bread from cv. Centauro; (21) negative control; (22) marker 100 bp.

Table 1. Electrophoretic Characteristics of the Microsatellites Examined

microsatellite	fragment size in durum wheat cv. Arcangelo (bp)	fragment size in common wheat cv. Pandas (bp)
	Monomorphic	
BARC008	250	250
GWM186	240	240
WMC41	160	160
WMC245	150	150
	Polymorphic in Length	
BARC006	320	300
GWM174	210	230
WMC167	160	150
	Polymorphic for Presence/Absence	
GWM52	390	190, 390
GWM194	no amplification	140, 200
GDM111	no amplification	210

one band of about 240 base pairs that, as expected, did not distinguish common wheat from durum.

To evaluate the effectiveness of D-genome specific wheat microsatellites in distinguishing common and durum wheat, the DNA extracted from semolina cv. Arcangelo, from flour samples cv. Pandas and cv. Centauro, and from Pandas bread, was used for a screening performed with nine selected microsatellite primers, chosen for being localized on D-genome according to literature data (*23*−*25*). Moreover, the primers were selected based on reported data regarding the dimensions of the amplified fragments. Primers in which amplicons were expected to range from 200 to 300 base pairs were chosen. The aim of the screening was to select the microsatellite targets expressing clearer profiles (possibly one single band) to effectively distinguish common wheat from durum wheat.

The obtained results, reported in **Table 1**, indicated that not all the primers amplified repetitive sequences localized exclusively on the D-genome. In fact, the majority of the primers

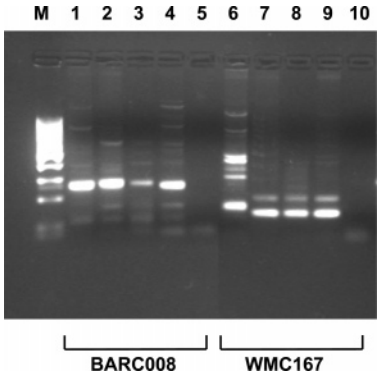

Figure 3. Agarose gel (2.5%) loaded with (from left to right): molecular weight marker 100 bp; amplification products of primer BARC008 with (1) semolina from durum wheat cv. Arcangelo; (2) flour from soft wheat cv. Pandas; (3) bread from soft wheat cv. Pandas; (4) flour from soft wheat cv. Centauro; (5) negative control; (6–10) the same samples in the same order amplified with primer WMC167.

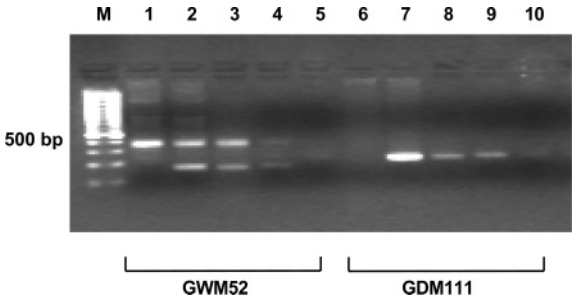

Figure 4. Amplification profile of primer GWM52 with (from left to right): (1) semolina from durum wheat cv. Arcangelo; (2) flour from soft wheat cv. Pandas; (3) commercial soft wheat pasta; (4) bread from soft wheat cv. Pandas; (5) negative control; (6–10) the same samples in the same order amplified with primer GDM111. Molecular weight marker 100 bp was loaded at the left side.

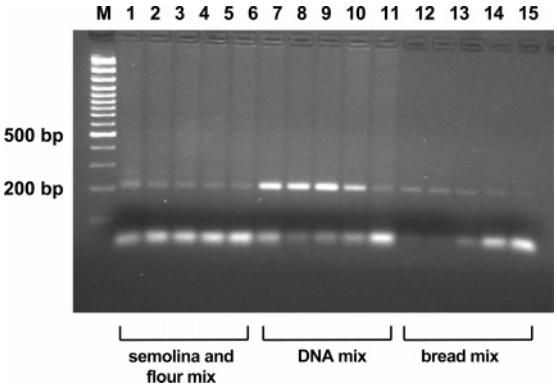

Figure 5. Amplification profile of primer GDM111 with (from left to right): mixtures of semolina of durum wheat cv. Appulo and flour of soft wheat cv. Pandas in the ratios (1) 70:30, (2) 80:20, (3) 90:10, (4) 95:5, and (5) 97:3; mixtures of DNA extracted from semolina of durum wheat cv. Appulo and flour of soft wheat cv. Pandas in the ratios (6) 70:30, (7) 80:20, (8) 90:10, (9) 95:5, and (10) 97:3; breads prepared from durum wheat cv. Appulo and from soft wheat cv. Pandas in the ratios (11) 70:30, (12) 80:20, (13) 90:10, (14) 95:5, and (15) 97:3. Molecular weight marker 100 bp was loaded at the left side.

(seven out of the nine examined) supplied for an amplification product in both durum and common wheat flours and breads indicate that the target sequence was localized also in the genomes A and/or B. Three of these seven primers did not distinguish *T. aestivum* and *T. turgidum*, i.e., they were

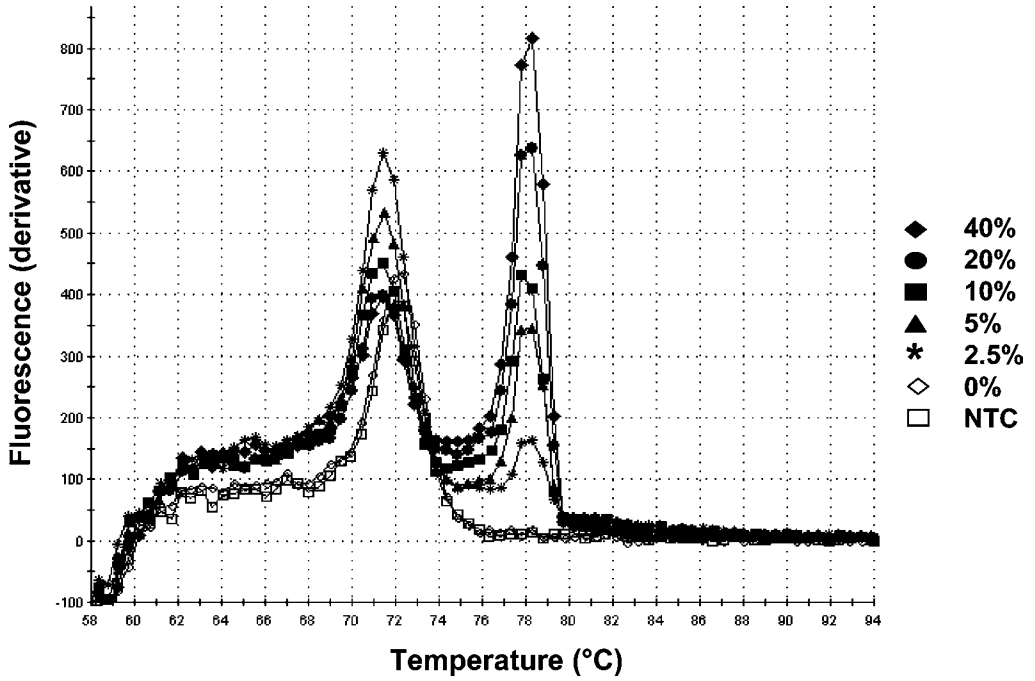

Figure 6. Dissociation curve (mean of three replicates) of real-time PCR, based on primer GDM111, assessed on mixtures of durum wheat (cv. Appulo) semolina with various percentages of soft wheat (cv. Pandas) flour. NTC = no template control.

monomorphic, while the other four primers were polymorphic between the two species and therefore enabled their distinction. However, these primers were not considered optimal to set up a method of detection of soft wheat since they showed a polymorphism of the amplification product regarding either its length or the intensity of its fluorescence, which could lead to doubtful results during the subsequent applications of the method. Unquestionable polymorphism of the type "presence/absence" of the amplified fragment is preferred when a method of legal value is established.

In **Figure 3** it is possible to observe the amplification profile of a primer showing length polymorphism between durum and soft wheat (WMC167), and an example of monomorphic pattern, due to primer BARC008. Primer GWM52 showed a more interesting profile (**Figure 4**), with a band sized 190 bp present in soft and absent in durum wheat, but its pattern was accompanied by another monomorphic band of 390 bp, so it was not considered optimal for our purposes.

Only two of the primers examined exclusively amplified soft wheat DNA, one leading to a pattern composed of multiple bands (GWM194) and the other optimally amplifying a single fragment (GDM111). The profile of the latter is reported in **Figure 4** and was chosen to set up the method of detection of soft wheat.

The primer GDM111 was tested over the whole sample set, comprising a total number of five pure durum wheat semolinas (from five single cultivars), two pure soft wheat flours, one commercial flour, one commercial semolina, and the corresponding nine breads, as well as a soft wheat pasta sample. The results confirmed that an amplicon of 210 base pairs was present solely in samples derived from soft wheat. Each tested durum wheat cultivar gave no amplification. The sample of pasta gave an amplified fragment with higher fluorescence, compared to that from soft wheat bread, indicating a better amplification level, due to the minor degree of degradation with respect to bread (**Figure 4**).

The commercial samples of flour and semolinas, usually derived from a mixture of grains of many cultivars, were

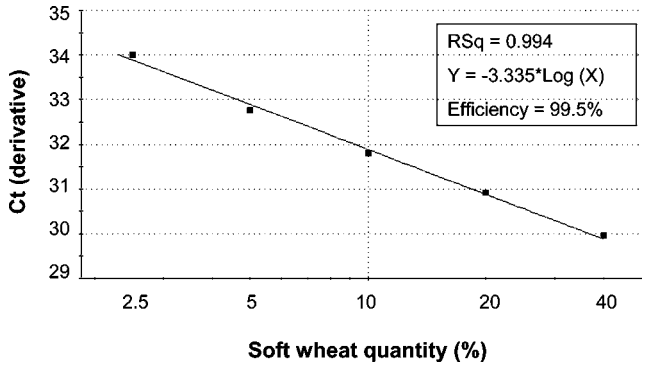

Figure 7. Real-time PCR standard quantification curve (mean of three replicates) of mixtures of durum wheat (cv. Appulo) semolina with 2.5, 5, 10, 20, and 40% soft wheat (cv. Pandas) flour. Primer GDM111 was used.

analyzed to verify the effect of the known intervarietal length polymorphism of the repetitive DNA sequences (*27*−*30*) on the result of the amplifications. Gel electrophoresis was used to check the differences in length of the PCR-generated products. No relevant smear was observed since the length variants did not differ more than a few base pairs and similar differences in allele size are difficult to resolve on agarose gels with ethidium bromide staining (*24*, *31*).

To quantify the threshold of the amount of soft wheat detectable by means of a PCR based on microsatellite GDM111, mixtures of DNA extracted from durum wheat semolina cv. Appulo and soft wheat flour cv. Pandas in the ratios 70:30, 80:20, 90:10, 95:5, and 97:3 were subjected to amplification. Mixtures of durum wheat semolina and soft wheat flour in the same ratios were also prepared, as well as the corresponding breads, the latter made at a bakery. DNA extraction and subsequent PCR were then performed from all the samples, with the aim to assess the threshold not only in DNA mixtures but also directly in flours and breads. Other authors (*32*), in fact, while setting up methods of analysis of food DNA for GMO

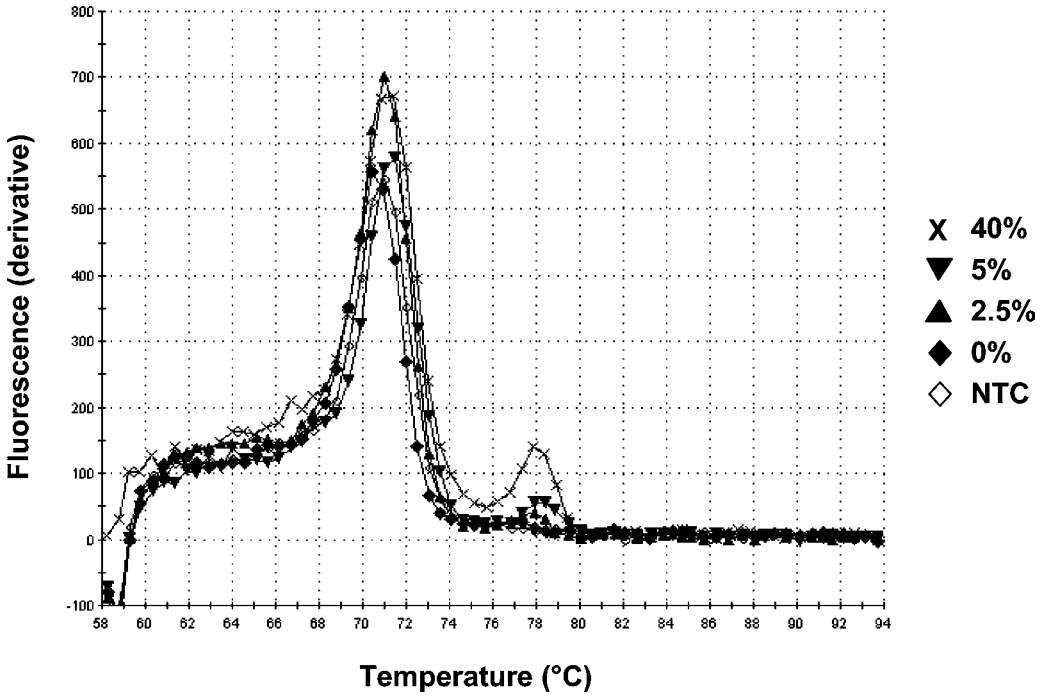

Figure 8. Dissociation curve (mean of three replicates) of real-time PCR, based on primer GDM111, assessed on DNA from breads of durum wheat cv. Appulo containing various percentages of soft wheat cv. Pandas. NTC = no template control.

detection purposes, observed significant differences between the results obtained by using a lab-prepared DNA mix and those achieved by starting from a food sample mixture, because of the effect of the extraction from a complex matrix. They concluded that the results determined solely using DNA blends should be further verified and a distinction should be stated between a "theoretical" threshold (determined by means of a DNA mix) and the "real" minimum detectable level (determined directly on food).

Also our results indicated a difference in the amplification of blended flours or breads with respect to the DNA mixtures. By amplifying primer GDM111 we were able to detect 3% soft wheat DNA in mixtures with durum wheat DNA. The same threshold was observed in detecting soft wheat in durum wheat semolina, while the threshold was raised to 5% in detecting soft wheat in baked products, i.e., in durum wheat breads. Furthermore, a marked difference was observed in the fluorescence intensity of the amplified fragments with a decreasing trend from DNA mix, flour mix, to blended bread (**Figure 5**).

Real-time quantitative PCR was also performed, based on the SYBR Green approach. This kind of method for real-time PCR does not require the synthesis of expensive probes and is very sensitive (*33*). An accurate preliminary phase of primer (GDM111) and DNA template concentration setting, and annealing temperature adjustment, was carried out to eliminate nonspecific amplifications and reduce primer-dimers. However, an increase of primer-dimer formation was observed when the amount of common wheat in the template decreased. To avoid measuring the signal due to dimers, fluorescence was detected at the end of an additional step at 76 °C added after each extension phase. In this way, it was possible to measure only the fluorescence of the specific product. The specificity of the PCR products was assessed on the basis of dissociation curve analysis. As known, a single peak is considered to be specific, while the appearance of multiple peaks would indicate nonspecific amplifications (*34*). Besides the primer-dimers, the dissociation curves (**Figure 6**) and agarose gel electrophoresis migrations showed a single amplicon of the expected size, indicating a good specificity of the reaction. As stated above, fluorescence due to primer-dimers, having a melting temperature slightly less than 72 °C, was not detected under our experimental conditions.

Furthermore, a standard quantification curve was assessed from real-time PCR analysis on DNA samples (three replicates) extracted from flour blends of common and durum wheat at various percentages. Standard curves showing a slope between approximately −3.1 and −3.6, with an Rsq value (Pearson coefficient) >0.985, are adequate for most applications requiring accurate quantification (minimum 90% reaction efficiency) (*35*). The standard curve (**Figure 7**) showed a slope of −3.335, and a RSq of 0.994, with an efficiency of 99.5%.

As for experiments carried out on bread samples, **Figure 8** shows the dissociation curves of durum wheat breads containing various percentages of soft wheat. It was possible to appreciate, with a better definition than in qualitative PCR experiments, the difference in fluorescence corresponding to different levels of soft wheat contamination. Anyway the fluorescence signal, particularly at the lowest common wheat concentrations, was rather low and not suitable to obtain a reliable standard curve with an acceptable efficiency. This was possibly due to a series of concurrent factors: (a) the presence of PCR inhibitors; (b) the lower yield of DNA from baked samples; (c) the high level of DNA degradation, as a consequence of the action of yeast nucleases during rising, and the combination of moisture and temperature connected with baking process; (d) a possible low efficiency of the primers used, known to be crucial in real-time PCR experiments (*6*, *36*, *37*). In any case, the dissociation curve showed that even 2.5% of common wheat in durum wheat bread was detectable, with a lower threshold than that observed in qualitative PCR while, as expected, 100% durum wheat bread produced no amplification, confirming the method selectivity.

In conclusion, the application of DNA microsatellite analysis to set up a method of detection of soft wheat was demonstrated. The results enabled selection of an efficient D-genome-specific repetitive DNA sequence to detect common wheat in semolina and durum wheat breads by qualitative PCR with a threshold

of 3 and 5%, respectively, lowered to 2.5% by real-time PCR. This is of major importance for some typical products recently protected with the European PDO mark such as Altamura bread, which should not contain soft wheat flour. The feasibility of an effective quantification of common wheat adulteration in semolina using real-time PCR was also demonstrated.

ACKNOWLEDGMENT

The Authors gratefully acknowledge Mr. Bartolo Digesù of Panificio Digesù (Bari, Italy) for accurate preparation of the examined breads.

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Received for review November 22, 2006. Revised manuscript received February 26, 2007. Accepted February 27, 2007. This research was supported by grants from "MIUR-Laboratorio di ricerca pubblico-privati", project AGROGEN.

JF063383E