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Literature and Bioinformatics Analyses of Wheat-specific Detection Methods

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Literature and Bioinformatics Analyses of Wheat-specific Detection Methods

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Executive Summary

In view of the recent necessity to perform testing for the detection of genetically modified common wheat (*Triticum aestivum*), the need arises for a taxon-specific method for this organism. However, no such method has yet been officially validated.

Multiple species of wheat exist on the market, such as common wheat, durum wheat, emmer wheat, etc. These plants have complex genomes, composed of different combinations (from diploid to hexaploid) of common sets of chromosomes. The specificity of a method then depends on which set of chromosome the targeted region is located, which increases the complexity of identifying methods specific to *Triticum aestivum*. Often, such methods were developed for the specific regulatory need of differentiating durum and common wheat (for example, in alimentary pasta labeling), with minimal concerns for non-specific detection of other plants.

This document summarises the review performed by the EU-RL GMFF, complemented with in-house bioinformatics analyses, in order to identify and characterise *Triticum aestivum*-specific detection methods that have been described in the scientific literature. Methods with apparent specificity (based on results shown and bioinformatics analyses) and promising performance (based on results shown) are highlighted and their primers and probe sequences reported. Those methods are the 'SS II-D' and ' SS II ex7' methods described in Matsuoka *et al.* (2012) and the 'wx012' method described in Iida *et al.* (2005), and they represent good candidates to uniquely identify common wheat in complex food samples.

Overview of common wheat (*Triticum aestivum)* taxon-specific methods in the scientific literature

The genome of the wheat family is complex, with four different types of genomes, symbolised by the letters A, B, D and G. In addition, some wheat species are diploid but many species on the market are stable polyploids with two sets (tetraploids) or three sets (hexaploids) of these genomes.

The major cultivated species of wheat are *Triticum aestivum* (common wheat), *T. spelta* (both hexaploids, with an AABBDD genome configuration), *T. durum and T. dicoccum* (both tetraploids, with an AABB genomic configuration). The selection of a target sequence for developing a detection/quantification taxon-specific method for common wheat is therefore very complex. In addition, both common wheat and durum wheat are widely used today, common wheat for foods such as bread, noodles and cakes and durum wheat for pasta products. A taxon-specific method should therefore be able to distinguish between these two highly similar species in addition to the other crop species generally present in food samples.

A search performed on the Internet identified the following articles describing methods for taxon-specific detection/quantification of wheat:

1. V. Terzi et al. (2003) *TaqMan PCR for Detection of Genetically Modified Durum Wheat.*

2. V. Terzi et al. (2003) *Development of Analytical Systems Based on Real-time PCR for Triticum Species-specific Detection and Quantitation of Bread Wheat Contamination in Semolina and Pasta.*

3. M. Iida et al (2005) *Development of Taxon-Specific Sequences of Common Wheat for the Detection of Genetically Modified Wheat.*

4. M. Hernández et al. (2005) *Real-Time Polymerase Chain Reaction Based Assays for Quantitative Detection of Barley, Rice, Sunflower, and Wheat.*

5. H. Yamakawa et al. (2007) *Specific Detection of Wheat Residues in Processed Foods by Polymerase Chain Reaction.*

6. Y. Matsuoka et al. (2012) *Development of Methods to Distinguish between Durum/Common Wheat and Common Wheat in Blended Flour Using PCR.*

7. EUROPEAN PATENT APPLICATION EP2180051A2 (2010) *Method for detecting and quantifying endogenous wheat DNA sequence*

Other three articles were brought to the attention of the EU-RL GMFF, in particular:

8. K. Brunner et al. (2009) *A Reference-gene-based Quantitative PCR Method as a Tool to Determine Fusarium Resistance in Wheat.*

9. S. Imai et al. (2012) *An Endogenous Reference Gene of Common and Durum Wheat for Detection of Genetically Modified Wheat.*

10. R. Alary et al. (2002) *Quantification of Common Wheat Adulteration of Durum Wheat Pasta Using Real-Time Quantitative Polymerase Chain Reaction (PCR).*

The methods described in these articles were analysed for their specificity for *Triticum aestivum* (common wheat). Based on this criterion, the list was narrowed down to four publications: Matsuoka *et al.* (2012), Iida *et al.* (2005), Imai *et al.* (2012) and Alary *et al.* (2012), which are further analysed below. The primers sequences for all the methods described in these articles are reported in Annex I.

The methods described in the remaining articles were showing non-specific amplification from DNA extracted from other *Triticum* species, rye, barley or soybean varieties and were not analysed further.

a. Matsuoka *et al.* 2012

Matsuoka *et al.* (2012) have developed conventional and real-time PCR detection methods based on the DNA sequence of the Starch Synthase II (SS II) gene, which is coded on wheat A, B and D genomes. Two set of primers and probes are described: one set (SS II ex7-U/L) targets a conserved region and allows detection of all wheat species, and the other primer pair (SS II-D 1769U/1889L) targets a sequence unique to the SS II-D variant (from the D genome) and can thus distinguish durum and common wheat.

The methods described in Matsuoka *et al*. have not been well characterised for intra-species and inter-species cross-reactions and have been developed so far only for qualitative testing.

According to the published results, the specificity of the methods was tested by end-point PCR and real-time PCR on DNA extracted from 5 common wheat samples, 3 durum wheat samples, barley, rye, buckwheat, rice, corn and soybean species. Amplification (114 bp amplicon) was detected with the first set of primers from common and durum wheat, and only from common wheat (121bp amplicon) with the second set of primers.

No amplification was observed from the other samples tested. Sensitivity studies performed by stepwise dilutions of common wheat DNA containing 20, 10, 1.0, 0.1, 0.05, 0.01, and 0.001 ng/uL indicated that the LOD of both methods could be close to 14,25 genome copies per reaction mixtures of real-time PCR.

The bioinformatics analyses on the two methods described in Matsuoka et al, 2012, based on the currently available information, confirm that the sets of primers and probe anneal to the wheat genome to produce the expected amplicons. Similar regions were

found in barley and *Brachypodium distachyon* (the available genome sequence closest to rye and oats), but with sufficient differences in the annealing regions to support specificity. The details of the analyses can be found in Annex II

b. Iida *et al.* 2005

Iida *et al.* designed a PCR methods targeting a gene specific for the D genome of wheat, waxy-D1 (Wx-D1, Genbank accession number of the mRNA: AF113844), thus allowing distinction between common and durum wheat. In common wheat, the *waxy* gene is present in three copies, Wx-A1, Wx-B1 and Wx-D1, found on chromosomes 7A, 4A, and 7D, respectively. A 1323 bp sequence of the Wx-D1 gene was sequenced to design different primers and probes. Among these, the authors selected and described the best performing pair.

The Iida *et al*. method has been designed for both qualitative and quantitative real-time PCR assays. It has been thoroughly tested on DNA extracted from seeds (not on food and feed samples) for specificity, genome copy number and intra-species variability, as summarized below:

	Haploid genomic copies	**% of target template in one reaction tube**
Specificity	Specific for common wheat (*T. aestivum*)	
Intra-species variability	No variability detected among 19 tested varieties	
N. of genome copies	One or two	
LOD	6.4-7.6 genome copies	0.5%
LOQ	15.1 genome copies	0.66%-0.78%
R^2 coefficient	0.996-0.999	
Slope	-3.4 and -3.7	
CV% of Ct values	1.015 to 2.475	
SD of Ct values	0.256-0.880	

Details of this reported performance can be found in Annex III

Bioinformatics analyses of the method described in Iida *et al* (see Annex IV) suggest that the primers described in the table 1 of the paper are incorrect, as they do not match the target sequence in the article's figure 2, and are different from the primers of the same name found in patent EP2180051 from the same authors. The patent's primer sequences do anneal correctly, and these sequences should be considered if this method is to be used.

This is most probably explained by a mistake in the primer sequences entered in the article's table 1. However, it is not possible to confirm this, and an uncertainty remains about what primers were used to generate the results described in Iida *et al* (2005), and summarized above.

c. Imai *et al.*, 2012

The Imai *et al*. article describes a conventional and real-time PCR targeting the wheat prolin-rich protein (PRP) gene. However, this target was specifically chosen to be common between common and durum wheat genomes. It is, therefore, not uniquely specific for *T. aestivum*, the host species of the genetically modified common wheat.

The methods was shown not to be able to detect other closely related plant species, including barley, rye, oats, rice, millet, sorghum, buckwheat, rapeseed and corn, consistent with our bioinformatics analyses (See Annex V).

d. Alary *et al.* 2002

The Alary et al. (2002) article describes a simplex and duplex real-time PCR targeting the puroindoline-b (pinb) gene, present in common wheat but absent in durum wheat, as well as the wheat lipid transfer protein (ltp) gene, present in both, to quantify common wheat adulteration of durum wheat pasta.

Given this purpose, the authors do not present an in-depth analysis of specificity and intra-species variability for the method. The article focuses on the optimisation of the real-time duplex PCR, the optimal dilution of the DNA extracts to avoid PCR inhibition and precision analysis for determination of common wheat contamination in pasta samples (contamination ranging from 1% to 10%). According to the authors, there are two copies of the ltp gene versus one copy of the pinb gene, but no data is presented and the results are unpublished.

The authors mention that puroindoline genes are located on the 5D chromosome of hexaploid wheat and are present in diploid species and absent in tetraploid species. It needs to be determined on which diploid wheat species the target sequence could possibly be detected.

It seems that testing across a wide range of commercial wheat to investigate copy number stability in *T. aestivum* and specificity of the method for common wheat against other closely related plant species still need to be performed. Moreover, the methods performance characteristics have not been thoroughly investigated in the article.

Conclusions

The review of the published results and the in-house bioinformatics analyses suggest that the methods described by Matsuoka *et al.* (2012) and Iida *et al.* (2005) represent good candidates to uniquely identify common wheat in complex food samples, as the amplicons are probably in a single copy in the wheat genome and no complete similarity with sequences in other genomes were detected.

The Iida *et al*. (2005) method has been thoroughly tested in the article on DNA extracted from seeds for specificity, genome copy number, intra-species variability and it has shown performances characteristics that seem to be in line with the ENGL performance acceptance criteria. The Iida et al. method is the only method that has been shown to be highly specific for common wheat and that has been sufficiently characterised both for qualitative and quantitative analysis. However, the sequence of the primers seem to be incorrect as they appear in Iida *et al.* (2005), and the corresponding patent's sequences (shown in Annex I) should be used instead.

The methods described in Matsuoka et al. (2005) appear also to be highly specific for common wheat only (SS II-D 1769U/1889L primer pair) or for both common wheat and durum wheat (SS II ex7-U/L primer pair) but have been developed so far only for qualitative testing. Further testing is required to evaluate the performance of the methods.

References

Rémi Alary, Arnaud Serin, Marie-Pierre Duviau, Philippe Jourdrier, Marie-Françoise Gautier (2002) *Quantification of Common Wheat Adulteration of Durum Wheat Pasta Using Real-Time Quantitative Polymerase Chain Reaction (PCR).* Cereal Chem. 79, 553-558 doi: 10.1094/CCHEM.2002.79.4.553

Kurt Brunner, Maria P. Kovalsky Paris, Guadalupe Paolino, Hermann Bürstmayr, Marc Lemmens, Franz Berthiller, Rainer Schuhmacher, Rudolf Krska, Robert L. Mach (2009) *A Reference-gene-based Quantitative PCR Method as a Tool to Determine Fusarium Resistance in Wheat.* Anal Bioanal Chem 395, 1385–1394 doi: 10.1007/s00216-009-3083-3

EUROPEAN PATENT APPLICATION EP2180051A2 (2010) *Method for detecting and quantifying endogenous wheat DNA sequence*

Marta Hernandez, Teresa Esteve, Maria Pla (2005) *Real-Time Polymerase Chain Reaction Based Assays for Quantitative Detection of Barley, Rice, Sunflower, and Wheat.* J. Agric. Food Chem. 53, 7003-7009. DOI: 10.1021/jf050797j

Mayu Iida, Satomi Yamashiro, Hirohito Yamakawa, Katsuyuki Hayakawa, Hideo Kuribara, Takashi Kodama, Satoshi Furui, Hiroshi Akiyama, Tamio Maitani, Akihiro Hino (2005) *Development of Taxon-Specific Sequences of Common Wheat for the Detection of Genetically Modified Wheat.* J. Agric. Food Chem. 53, 6294-6300. DOI: 10.1021/jf0505731

Shinjiro Imai, Keiko Tanaka, Yasuyuki Nishitsuji, Yosuke Kikuchi, Yasuyuki Matsuoka, Shin-Ichiro Arami, Megumi Sato, Hiroyuki Haraguchi, Youichi Kurimoto, Junichi Mano, Satoshi Furui, Kazumi Kitta (2012) *An Endogenous Reference Gene of Common and Durum Wheat for Detection of Genetically Modified Wheat.* Food Hyg. Saf. Sci. 204 Vol. 53 203-210

Yasuyuki Matsuoka, Shin-Ichiro Arami, Megumi Sato, Hiroyuki Haraguchi, Youichi Kurimoto, Shinjiro Imai, Keiko Tanaka, Junichi Mano, Satoshi Furui, Kazumi Kitta (2012) *Development of Methods to Distinguish between Durum/Common Wheat and Common Wheat in Blended Flour Using PCR.* Food. Hyg. Saf. Sci. 196 Vol. 53, 195-202

Valeria Terzi, Barbara Ferrari, Franca Finocchiaro, Natale Di Fonzo, Antonio Michele Stanca, Carmen Lamacchia, Johnathan Napier, Peter R Shewry, Primetta Faccioli (2003) *TaqMan PCR for Detection of Genetically Modified Durum Wheat.* Journal of Cereal Science 37, 157-163. DOI: 10.1006/jcrs.2002.0489

Valeria Terzi, Mauro Malnati, Martino Barbanera, A.Michele Stanca, Primetta Faccioli (2003) *Development of Analytical Systems Based on Real-time PCR for Triticum Species-specific Detection and Quantitation of Bread Wheat Contamination in Semolina and Pasta.* Journal of Cereal Science 38, 87–94. DOI: 10.1016/S0733-5210(02)00138-8

Hirohito Yamakawa, Hiroshi Akiyama, Yumi Endo, Kiyoko Miyatake, Kozue Sakata, Shinobu Sakai, Masatake Toyoda, Atsuo Urisu (2007) *Specific Detection of Wheat Residues in Processed Foods by Polymerase Chain Reaction.* Biosci. Biotechnol. Biochem. 71, 2561-2564. doi:10.1271/bbb.70251

Annex I – Summary of the primers sequences from the selected publications described in the document

Matsuoka et al. 2012 *T.aestivum* and *T. durum* specific	Forward primer:	SS II ex7-U	5'-GGATGGAAATCTGGTGTTT-3'
	Reverse primer:	SS II ex7-L	5'-ACCATAATGGACCGAGTGTAC-3'
	Probe:	SS II ex7-T82	FAM-5'-CTCCTGCCTGTCTATCTGAAAGCAT-3'-TAMRA
Matsuoka et al. 2012 *T.aestivum*-specific	Forward primer:	SS II-D 1769U	5'-CACCATCAGTGAAGGAATGAATG-3'
	Reverse primer:	SS II-D 1889L	5'-GGCGATATTTGGTACCTAATTGAAG-3'
	Probe:	SS II-D-1797T	FAM-5'-TACCCGATCGACCGTTTTGCC-3'-TAMRA
Iida et al. 2005[1] *T.aestivum*-specific	Forward primer:	wx012-5'	5'-GGTCGCAGGAACAGAGGTGT-3'
	Reverse primer:	wx012-3'	5'-GGTGTTCCTCCATTGCGAAA-3'
	Probe:	wx012-T	FAM-5'-CAAGGCGGCCGAAATAGGTTGCC-3'-TAMRA
Imai et al. 2012 *T.aestivum* and *T. durum* specific	Forward primer:	PRP8F	5'-GCACCCATGATGAGTACTACTATTCTGTA-3'
	Reverse primer:	PRPds6R	5'-TGCAAACGAATAAAAGCATGTG-3'
	Probe:	PRP-Taq5	FAM-5'-CTGTGCACATGACTCAGTTGTTCTTTCGTG-3'-TAMRA

[1] Note: sequences taken from the patent, not the article

Annex II – Bioinformatics analyses of the Matsuoka et al. (2012) methods

Method 1: SS II ex7

Wheat

The amplicon described in figure 1A of the article was found in a single copy in the wheat genome, according to the currently available genome sequences.

Barley

There is a region in barley with some similarity with the proposed method, but there are some differences that may be sufficient to avoid amplification:

>EG:7HS dna:chromosome chromosome:030312v2:7HS:1:43655320:1

```
       1 GGGATGGAAATCTGGTGTTTATTGCAAATGATTGGCACACGGCACTCCTGCCTGTCTATC 60
         ||||||||||||||| ||                         ||||||||||||||||
13607323 GGGATGGAAATCTGGTCTTCATTGCAAATGATTGGCACACGGCACTCCTGCCTGTCTATC 13607382

      61 TGAAAGCATATTACAGGGACCATGGTTTGATGCAGTACACTCGGTCCATTATGGT 115
         ||||||||                          |||| ||| ||| |||||||
13607383 TGAAAGCATATTACAGGGACCATGGTTTGATGCAATACAGTCGCTCCGTTATGGT 13607437
```

Rye and Oat

Genome sequences for Rye and Oat are not yet available. No sequence similar to the amplicon was found in Genbank for these two species. Searching the genome from Brachypodium distachyon, the closest species from Rye and Oat from which the genome is available, finds only one possible similar region, once again with many differences, and only a partial binding sequence for the reverse primer:

>EG:1 dna:chromosome chromosome:v1.0:1:1:74834646:1

```
1        GGGATGGAAATCTGGTGTTTATTGCAAATGATTGGCACACGGCACTCCTGCCTGTCTATC 60
         ||||||||||||||||||                         || || || |||||||
43332859 GGGATGGAAATCTGGTGTTCATCGCAAATGATTGGCACACTGCACTACTACCAGTCTATC 43332800

61       TGAAAGCATATTACAGGGACCATGGTTTGATGCAGTACAC... 100
         ||||||||                          ||||||
43332799 TGAAAGCATATTACAGAGACCATGGCTTGATGCAGTACAC... 43332760
```

Method 2: SS II-D

Wheat

The amplicon described in figure 1B of the article was found in a single copy in the wheat genome, according to the currently available genome sequences. A second region showed some similarity, but there are some differences that may be sufficient to avoid amplification:

```
3     …CCATCAGTGAAGG-AATGAATGTCGGGTACCCGATCGACCGTTTTGCCCAACGTCGGGTT 61
       |||||||||||| ||||||||     ||||||||||||||||||||
1146 …CCATCAGTGAAGGGAATGAATGTCGGGTACCCGATCGACCGTTTTGCCCGACGTCAGGTT 1205

62    TACCCGCCCTATAGATCCGAATAAGTAGTTCCTATCTTCAATTAGGTACCAAATATCG… 119
                                         | ||| ||| ||||||||||||
1206 TACCCGCCCTGTAGGTCCGAATAAGTAGTTCCTATCCTCAGTTAAGTACCAAATATCG… 1263
```

Barley

There is a region in barley with some similarity with the proposed method, but there are most probably too many differences for amplification:

```
       7 …CAGTGAAGGAATGAATGTCGGGTACCCGATCGACCGTTTTGCCCAACGTCGGGTTTACCC 66
          ||  |||||||||||||     |||| ||| | |||||| ||
28920061 …CAAAGAAGGAATGAATGTCGGGTACC-GATAGGCCGTTTGGCTTGACGTCGGGTTTACCT 28920119

      67 GCCCTATAGATCCGAATAAGTAGTTCCTATCTTCAATTAGGTACCAAATATCGCC 121
                                       | | ||||| |||| ||| ||||||
28920120 GCCCTGTAGATCCGAATAAGTAGTTCCTATCCTTAATTAAGTACAAAA-ATCGCC 28920173
```

Rye and Oat

No sequence similar to the amplicon was found in Genbank for these two species, nor in the genome from *Brachypodium distachyon*.

Annex III – Details of the performance of the Iida et al. (2005) method, as reported in the publication.

Specificity

The selected primers and probes were evaluated by both qualitative and quantitative PCR on DNA extracted from the plant species listed in the following table that also summarises the results obtained:

Species	Qualit.	Quant.
barley	-	-
rye	-	-
oats	-	-
Italian millet (awa)	-	-
common millet	-	-
soybeans	-	-
buckwheat	-	-
Rice (Eleven varieties for qual.)	-	-
oilseed rape	-	-
maize	-	-
sesame	-	
4 varieties of durum wheat	-	
Kidney beans		-
Chickpeas		-
Common wheat (19 different varieties)	X	X

In qualitative PCR analysis the expected 102 bp band was observed from the common wheat samples and there were no amplification products detected from any of the other species tested. No amplification other than from common wheat was observed for the real-time PCR analyses. The DNA sequence from each PCR product corresponded to the expected genomic DNA sequence.

Intra-species variability

The intra-species variability of the Wx012 region was tested among 19 different wheat varieties.
Results: in qualitative PCR analysis, PCR products of identical sizes and equivalent intensities were obtained and no additional bands were observed in the tested varieties. Similar amplification plots with Ct values showing slight variability (Ct ranging from 27.56 to 28.50) were observed by real-time PCR analysis. These results suggest that the copy number of the Wx012 region was considered to be identical among the varieties tested.

Genome copy number

Genome copy number of the Wx-D1 gene was investigated by Southern blot analyses on DNA digested with BamHI, EcoRI and FbaI restriction enzymes and hybridized with a 444bp DNA fragment of the Wx-D1 gene.
Results: the hybridized bands indicated that the Wx012 region of endogenous DNA could be present in one or two copies on the D genome of wheat.

LOD

The sensitivity of the Wx012 system was tested by performing PCR amplification three times at ten dilutions of wheat genomic DNA (ranging from 0-150 ng of DNA as a template).
Results: The limit of detection was calculated at 6.4-7.6 genome copies, assuming a haploid genomic size of common wheat corresponding to 16.5-19.5 pg. The detection limit of the Wx012 system is therefore theoretically around 0.5% of target template in one reaction tube.

LOQ

The LOQ corresponded to 15.1 genome copies. The LOQ could be in the range of 0.66%-0.78% of target DNA template using 50 ng of DNA template in one PCR tube.

Amplification Efficiency

The slope was shown to range between -3.4 and -3.7. The R2 coefficient was calculated to be 0.996 - 0.999.

Precision

Quantitative PCR was run in triplicate with five dilutions of common wheat DNA ranging between 0.1-300 ng/uL**.**
Results: The CV (%) of the Ct values ranged between 1.015% to 2.475% and the relative SD ranged from 0.256 to 0.880 respectively.

Annex IV – Bioinformatics analyses of the Iida et al. (2005) methods

The primers from Iida et al were compared to the 3' terminal sequence of Wx-D1 (shown on figure 2 of the article). This sequence was extracted from the article figure as no corresponding Genbank record was found.

Alignment analyses showed that the set of primers and probe do not correspond perfectly to the published sequence, as both the wx012-5' primer and the wx012-T probe have a one base difference compared to the target sequence.

In the patent EP2180051, from the same authors, the sequences of the same primers are shown; however, despite having the same name, the sequences differ. The sequences from the patent match perfectly with the sequence shown in figure 2 of the article. Also, Wx012-5' is one base longer in the patent compared to the article.

This can be seen in the alignment of the sequence in figure 2 of the Iida *et al*. (2005) article with the primers sequences found in the same article, and the equivalent primer sequences found in the patent:

```
                            wx012-5'              wx012-T
Primers (article):     GTCGCGGGAACAGAGGTG  CAAGGCGGCCGAA
Figure 2 sequence: TCGGTCGCAGGAACAGAGGTGTTCAAGGCGGCCGAA
Primers (patent):     GGTCGCAGGAACAGAGGTG  CAAGGCGGCCGAA



Primers (article): ATAAGTTGCC
Figure 2 sequence: ATAGGTTGCCGCCTGCGGCGGAATCGCCACCCAC
Primers (patent):  ATAGGTTGCC

                                              wx012-3'
Primers (article):                 TTTCGCAATGGAGGAACACC
Figure 2 sequence: CGTGAAGTTCACCGTTTCGCAATGGAGGAACACCTA
Primers (patent):                  TTTCGCAATGGAGGAACACC
```

In addition, it should be noted that, comparing the fragment of the sequence in the article that corresponds to the amplicon and the currently available genomic sequence of *Triticum aestivum*, there is a one base gap that corresponds to the last base of the wx012-3' primer, as shown below:

```
1   GGTCGCAGGAACAGAGGTGTTCAAGGCGGCCGAAATAGGTTGCCGCCTGCGGCGGAATCG 60
    ||||||||||||||||||   ||||||||||||||||||||||
456 GGTCGCAGGAACAGAGGTGTTCAAGGCGGCCGAAATAGGTTGCCGCCTGCGGCGGAATCG 397

61  CCACCCACCGTGAAGTTCACCGTTTCGCAATGGAGGAACACC 102
                          ||||||||||||||||||
396 CCACCCACCGTGAAGTTCACCG-TTCGCAATGGAGGAACACC 356
```

Annex V – Bioinformatics analyses of the Imai et al. (2012) method

The figure 2 of the Imai *et al*. (2012) article shows the alignment of the expected amplicon sequence and the equivalent region of the Barley genome. The same differences were observed in our analyses, and no other sequences were found in the databases, from other species, that had fewer mismatches than the Barley sequence.

In addition, it should be noted that, comparing the fragment of the sequence in the article that corresponds to the amplicon and the currently available genomic sequence of *Triticum aestivum*, there is a one base mismatch in the probe sequence (identical to the mismatch seen in the barley sequence), as seen in the alignment of the amplicon sequence from the article with the available *Triticum aestivum* genome sequence (the primers and probe sequences are highlighted in green):

```
1   GCACCCATGATGAGTACTACTATTCTGTAATTATCATTTGCGTGTTGGTATATGTTCATC 60
    ||||||||||||||||||||                                      |
184 GCACCCATGATGAGTACTACTATTCTGTAATTATCATTTGCGTGTTGGTATATGTTCATC 243

61  TGTGCACATGACTCAGTTGTTCTTTCGTGTAGATACACATGCTTTTATTCGTTTGCA 117
    |||||||||||||| ||||||||||||||      ||||||||||||||||||||||
244 TGTGCACATGACTCGGTTGTTCTTTCGTGTAGATACACATGCTTTTATTCGTTTGCA 300
```

It is unclear which of the two sequences is correct, and/or if the mismatch is a natural variation at this residue in the wheat genome.

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Abstract

In view of the recent necessity to perform testing for the detection of genetically modified common wheat (Triticum aestivum), the need arises for a taxon-specific method for this organism. However, no such method has yet been officially validated.

This document summarises the review performed by the EU-RL GMFF, complemented with in-house bioinformatics analyses, in order to identify and characterise Triticum aestivum-specific detection methods that have been described in the scientific literature. Methods with apparent specificity (based on results shown and bioinformatics analyses) and promising performance (based on results shown) are highlighted and their primers and probe sequences reported. Those methods are the 'SS II-D' and ' SS II ex7' methods described in Matsuoka et al. (2012) and the 'wx012' method described in Iida et al. (2005), and they represent good candidates to uniquely identify common wheat in complex food samples.

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