Development and validation of a method for quantification of common wheat, durum wheat, rye and barley by droplet digital PCR

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Abstract

Food fraud is becoming a prominent topic in the food industry. Thus, valid methods for detecting potential adulterations are necessary to identify instances of food fraud in cereal products, a significant component of human diet. In this work, primer–probe systems for real-time PCR and droplet digital PCR (ddPCR) for the detection of these cereal species: bread wheat (together with spelt), durum wheat, rye and barley for real-time PCR and ddPCR were established, optimized and validated. In addition, it was projected to validate a molecular system for differentiation of bread wheat and spelt; however, attempts for molecular differentiation between common wheat and spelt based on the gene GAG56D failed because of the genetic variability of the molecular target. Primer–probe systems were further developed and optimized on the basis of alignments of DNA sequences, as well as already developed PCR systems. The specificity of each system was demonstrated on 10 (spelt), 11 (durum wheat and rye) and 12 (bread wheat) reference samples. Specificity of the barley system was already proved in previous work. The calculated limits of detection ($LOD_{95\%}$) were between 2.43 and 4.07 single genome copies in real-time PCR. Based on the “three droplet rule”, the $LOD_{95\%}$ in ddPCR was calculated to be 9.07–13.26 single genome copies. The systems were tested in mixtures of flours (rye and common wheat) and of semolina (durum and common wheat). The methods proved to be robust with regard to the tested conditions in the ddPCR. The developed primer–probe systems for ddPCR proved to be effective in quantitatively detecting the investigated cereal species rye and common wheat in mixtures by taking into account the haploid genome weight and the degree of milling of a flour. This method can correctly detect proportions of 50%, 60% and 90% wholemeal rye flour in a mixture of wholemeal common wheat flour. Quantitative results depend on the DNA content, on ploidy of cereal species and are also influenced by comminution. Hence, the proportion of less processed rye is overestimated in higher processed bread wheat and adulteration of durum wheat by common wheat by 1–5% resulted in underestimation of common wheat.

Keywords Cereal · Quantification · Food fraud · PCR · ddPCR

Introduction

Cereals and cereal products are a significant source of nutrition. In Germany, the consumption of cereal products averaged 81.7 kg per capita in 2017 and 2018 [1]. Cereal products are among the top 10 products in Europe with the highest risk of food fraud [2]. Food fraud is defined as a mismatch between food claims and actual food properties through deliberate misdeclaration or deliberate omission

of claims [3]. Furthermore, it refers to the substitution, manipulation, falsification and adulteration of individual components or of the entire food [2]. In cereal products, the partial or total substitution of value-added components (e.g. substitution of rye flour with cheaper wheat flour) is of particular importance as it represents a financial disadvantage for the consumer. However, in the legal assessment of these products of agricultural origin, adventitious presence as a result of cultivation, harvesting, transport and storage must always be taken into account (e.g. for durum wheat according to Regulation (EU) No. 2016/1238 a permitted proportion of 3%).

The tribus *Triticeae* evolved about 25 million years ago in the sweet grass family (*Poaceae*) [4]. Important cereal species in this tribus are common wheat (*Triticum aestivum*

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subsp. aestivum), rye (*Secale cereale*), barley (*Hordeum vulgare*) and durum wheat (*Triticum turgidum* subsp. *durum*) [5]. The tribus consists of the two subtribes: *Hordeineae* and *Triticineae*. *Hordeineae* gave rise to the genus *Hordeum* with the important representative *Hordeum vulgare*. The subtribe *Triticineae* first gave rise to the genus *Secale* (representative *Secale cereale*) and finally to the genera *Triticum* and *Aegilops* [4]. Starting with the original form *Triticum urartu* (diploid, genome AA), the tetraploid species *Triticum turgidum* arose in the genus *Triticum* through allopolyploidisation with *Aegilops speltoides* (diploid, genome SS), from which *Triticum turgidum* subsp. *dicoccon* (emmer, tetraploid, genome AABB) emerged through cultivation. Further cultivation produced *Triticum turgidum* subsp. *durum* (tetraploid, genome AABB) and further *Triticum turgidum* subsp. *turanicum* (kamut) [4, 6]. Hybridization of the species *Triticum turgidum* (genome AABB) with *Aegilops tauschii* (diploid, genome DD) resulted in the hexaploid *Triticum* species *Triticum aestivum* subsp. *spelta* (hexaploid, genome AABBDD) and *Triticum aestivum* subsp. *aestivum* (hexaploid, genome AABBDD) [7]. Another hybridization between *Secale cereale* (diploid, genome RR) and *Triticum aestivum* produced *Triticale* (octoploid, genome AABBDDRR) [8].

Enzyme-linked immunosorbent assay (ELISA) [9], chromatographic methods [9–13], near-infrared spectroscopy (NIRS) [14, 15] and real-time PCR [16–22] and other molecular techniques [23] were used for the specific detection of cereal species in food. Compared to other analytical parameters, such as proteins and fats, DNA is sufficiently stable, depending on the degree of processing, to be detected even in more processed foods [24]. Therefore, molecular methods based on chromosomal genes can potentially be used for quantification [21]. For the specific detection of rye, the TaqMan® PCR system, based on the single-copy gene for myo-inositol-1-phosphate synthase (MIPS), was developed by PEGELS et al. [17]. CASAZZA et al. were able to detect differences between durum and common wheat in the intron region of the β -tubulin gene on the B genome using the tubulin-based polymorphism method and developed an SYBR Green PCR method for the specific detection of durum wheat [18]. To identify common wheat, MATSUOKA et al. used the Starch Synthase II gene (wSSII-D), located on chromosome 7D [19, 25]. The barley system 1, based on the hordein gene and developed by ZELTNER et al., showed good specificity not only for *Hordeum vulgare*, but also for *Hordeum murinum* [21]. In addition, several systems claimed to be able to distinguish bread wheat from spelt [14, 16, 22].

Compared to real-time PCR, digital PCR (dPCR) allows absolute quantification of copy numbers [26] and is thus used in GMO quantification [27], species detection and quantification [28] and detection of various pathogens in food [26, 29]. A variant of dPCR is droplet digital PCR (ddPCR), in which the reaction mixture is partitioned into

about 20,000 smaller reactants (droplets) with an average volume of 0.89 nL [28]. The distribution of DNA in these partitions follows the Poisson distribution. This water-in-oil emulsion is amplified by an endpoint PCR in which each target molecule is amplified in its compartment separately. Hence, in the presence of target DNA in a droplet, amplification and an increase in fluorescence take place. By counting the droplets that show fluorescence and statistical conversion, the absolute copy number of the target DNA can be determined precisely and, in contrast to quantitative real-time PCR, independently from most parameters influencing the amplification efficiency [30]. This allows the determination of the amounts of grain species used. At this time, no method has been published for the quantitative determination of common wheat, durum wheat, rye and barley by ddPCR.

Materials and methods

Sample material

The Institute of Crop Plant Research and Genetics in Gatersleben (IPK Gatersleben, Germany) provided the reference material for the following cereal varieties: common wheat (13), durum wheat (12), spelt (11), barley (12), rye (12), triticale (1), einkorn (1), emmer (1), kamut (1) and buckwheat (1). Sample material for sunflower and pumpkin seeds, whole rye flour, common wheat and durum wheat semolina was obtained from local grocery stores. The other flours were provided by SchapfenMühle GmbH & Co. KG (Ulm, Germany). The complete overview of the sample material is presented in the Electronic Supplementary Material (Supp. Tables 1, 2).

For validation of quantitative analyses, flour blends were prepared from whole rye flour with whole wheat flour (mixture named RW) and rye flour type 997 (i.e. 997 mg ash per 100 g dry mass flour) with wheat flour type 550 (i.e. 550 mg ash per 100 g dry mass flour) (mixture named RoWe) in the mass proportions: 50/50, 60/40 and 90/10, with the first number being the rye proportion and the second number being the common wheat proportion. Semolina blends of durum semolina with common wheat semolina were further produced in mass proportions of 99/1, 98/2, 97/3, and 95/5, with the first number being the durum portion and the second number being the common wheat portion. The flour and semolina were dried to a mass consistency at 70 °C before being homogeneously mixed with a mortar and pestle. Semolina from the different species was finely powdered with mortar and pestle before further used. The semolina mixtures were finally made from ground material by mixing in a Grindomix GM200 (Retsch, Haan, Germany) with repeated scraping.

Primers and probes

Primers and probes (ZENTM Double-Quenched Probes, reporter: 6-carboxy-fluorescein; quencher: Iowa Black® FQ) were supplied by Integrated DNA Technologies (Coralville, USA) and were designed with the PrimerExpress® software version 2.0 (Thermo Fisher Scientific, Waltham, USA). Table 1 shows the primers and probes for each cereal species-specific PCR system. The complete list of primers and probes used is shown in the Electronic Supplementary Material (Supp. Tables 3, 4).

Sample preparation and DNA extraction

Comminution of the reference material was performed by FastPrep FP120 (Qbiogene, Illkirch, France) using Metal Lysing Tubes, 2 mL with grinding cylinder (MP Biomedicals, Eschwege, Germany). Extraction was performed using a modified CTAB extraction [31]. From the minced material, 200 mg was suspended with 1000 µL of CTAB extraction buffer [c (CTAB) = 20 g/L, c (NaCl) = 1.4 mol/L, c (TRIS) = 0.1 mol/L, c (Na₂EDTA) = 0.02 mol/L, pH 8.0], and 100 µL of α-amylase [from *Bacillus licheniformis*, 880 U/mL] (Sigma, Munich, Germany) was added. Samples were incubated for 45 min at 90 °C with shaking. Then, 10 µL RNase A [c = 30 mg/mL] (Macherey–Nagel, Düren, Germany) was added and incubated again for 45 min at 65 °C with shaking. Further workup was performed as described in [32]. In brief, 10 µL Proteinase K solution (c = 20 mg/L) was added, and the samples were incubated at 60 °C under agitation overnight and centrifuged for 10 min at 12,000×g afterwards. 0.7 mL of chloroform was added to the supernatant, shaken vigorously and centrifuged at 19,000×g for 10 min. The upper phase was transferred into a new vial. Two

volumes of CTAB precipitation buffer [c (CTAB) = 5 g/L, c (NaCl) = 0.04 mol/L] were added and incubated 60 min at room temperature. The samples were centrifuged for 5 min at 12,000×g. The pellet was resuspended in 350 µL NaCl solution [c (NaCl) = 1.2 mol/L] and 350 µL of chloroform were added; the samples were vigorously shaken and centrifuged for 10 min at 19,000×g. 0.6 vol of isopropanol was added to the upper phase for nucleic acid precipitation. After 20-min incubation at room temperature, the samples were centrifuged for 10 min at 12,000×g. The resulting pellet was washed with 500 µL ethanol solution (c = 70%) and resolved in 100 µL 0.1×TE buffer [c (TRIS) = 1 mmol/L, c (Na₂EDTA) = 0.1 mmol/L, pH 8.0] and stored as stock solution. Determination of the concentration and purity of dsDNA from the obtained DNA extracts was performed by measuring the optical density at 260 and 280 nm using a ScanDrop photometer (Analytik Jena, Jena, Germany). DNA purity was considered sufficiently if OD260/OD280 was at least 1.8.

DNA-sequencing

Selected samples were sequenced to verify the target regions in the DNA sequence. Three reference samples (R 01/1, R 12/1, R 14/1) for rye were sequenced with primers Rmips01-fw and Rmips01-rv. To screen for a wheat- and spelt-specific system for the gene wSSII-D (AB201447.1), three spelt samples (SP 03/1, SP 13/1, SP 18/1) and three common wheat samples (TRI 03/1, TRI 15/1, TRI 20/2) were sequenced with primers Wss2d01-fw and Wss2d01-rv. Sequencing for the durum wheat-specific β-tubulin gene (MG852148.1) of *Triticum durum* was performed with primers Dtub05-fw and Dtub03-rv on the reference samples for durum wheat (DU 01/1, DU 08/1, DU 18/1), common wheat (TRI 15/1, TRI

Table 1 Overview of primers and probes (probes are underlined) used for real-time PCR and ddPCR. For further information, see Electronic Supplementary Material Supp. Table 3

Name of primers & probes (probes underlined)	Sequence 5' → 3'	Target species (name of system)	Gen Accession Number (NCBI) Lit	Lit
<i>Rmips02-fw</i>	TATTTTCGGCTCTTTGCGACAT	Rye (<i>Rmips02</i>)	This work	This work
<i>Rmips02-rv</i>	CACCCTCCTCCCTCCACAT			
<i>Rmips02-p</i>	TAGCTTCTCATGGACTCCATGGTGTGG			
<i>Dtub03-fw</i>	TCATGGTGCAATCTATATATCCTTCTG	Durum wheat (<i>Dtub03</i>)	MG852148 This work	This work
<i>Dtub03-rv</i>	TGGGTGGATTGCATGGATTG			
<i>Dtub03-p</i>	TTGTCCGCCACCAAATAAGTAGCATAAG			
<i>Wss2d01-fw</i>	CACCATCAGTGAAGGAATGAATG	Common wheat & spelt (<i>Wss2d01</i>)	AB201447 [19]	[19]
<i>Wss2d01-rv</i>	GGCGATATTTGGTACCTAATTGAAG			
<i>Wss2d01-p</i>	TACCCGATCGACCGTTTGGC			
<i>HV-F</i>	CTCCTTGCTTATCCAATTTCTTTTG	Barley (<i>HV</i>)	X84368.1 [21]	[21]
<i>HV-R</i>	CTAAGAAGAAACACGGGAAGAAYAA			
<i>HV-S</i>	TGGCAAACCTGCACCTKTCCAACCGAT			

17/1) and kamut (TRI 19,236). Ten reference samples of common wheat (TRI 01/1, TRI 02/1, TRI 03/1, TRI 04/1, TRI 05/1, TRI 16/1, TRI 17/1, TRI 18/2, TRI 19/2, TRI 20/2) and seven reference samples of spelt (SP 05/1, SP 08/1, SP 09/1, SP 13/1, SP 15/1, SP 17/1, SP 18/1) were sequenced with primers Sglia01-fw and Sglia03-rv for alignment with published gene sequences for the γ -gliadin gene (*Triticum aestivum*—AF144104.1; *Triticum spelta*—AF120267.1) to test whether or not this gene could distinguish spelt from common wheat, based on a 9-bp insertion in the γ -gliadin gene of common wheat [16].

Conventional PCRs were performed in a reaction mixture of 25 μ L total volume consisting of 0.125 μ L Hot Star Taq DNA Polymerase [$c = 5$ U/ μ L] (Qiagen, Hilden, Germany), 2.5 μ L 10 $\times$ PCR buffer containing c (MgCl_2) = 15 mmol/L (Qiagen, Hilden, Germany), 0.5 μ L dNTP mix [c (dATP; dCTP; dGTP; dTTP) = 200 μ mol/L each] (Roche Diagnostics, Mannheim, Germany), 500 nmol/L of each primer and 5 μ L template DNA [c (DNA) = 100 ng/ μ L]. Reactions were performed in the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Darmstadt, Germany) using an initial denaturation step at 95 °C for 15 min followed by 45 cycles of 30 s at 95 °C, 30 s annealing at 5 °C below the melting temperature calculated for the primers and 30 s at 72 °C. A final elongation step was performed for 7 min at 72 °C. Gel electrophoresis was performed in a 2% agarose gel in TAE buffer with 5 μ L PCR product using a 50-bp fragment length marker (Gene Ruler™, Thermo Scientific, Waltham, USA). Bands were stained with 1% ethidium bromide solution for visualization.

The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing of the purified PCR products was performed with the BigDye™ Terminator V 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) using the same primers as those in conventional PCR. The reaction conditions of the sequence reaction were chosen analogously to [32]. The annealing temperature of the primers was 5 °C below the melting temperature; see Electronic Supplementary Material (Supp. Table 3). The products were analyzed in the ABI Prism 310 Genetic Analyzer sequencer (Applied Biosystems, Darmstadt, Germany) and evaluated using the Sequence Navigator software version 1.0.1 (Applied Biosystems, Darmstadt, Germany). With Basic Local Alignment Search Tool (BLAST), the sequences were compared with those in the GenBank® database [33].

Real-time PCR

For real-time PCR, a total volume of 25 μ L per reaction tube was chosen with a volume of template of 5 μ L [c (DNA) = 20 ng/ μ L], 12.5 μ L TaqMan® 2 $\times$ Universal PCR Mix (Applied Biosystems, Darmstadt, Germany) and the

corresponding volume of forward and reverse primer and probe [$c = 10$ μ M]. A final concentration of 300 nM was chosen for the primers HV-F, HV-R, Rmips02-fw and Rmips02-rv, and a concentration of 600 nM was chosen for Dtub03-fw, Dtub03-rv, Wss2d01-fw and Wss2d01-rv in the PCR assay. For the HV-S probe, the final concentration was 150 nM, and for all other probes, 100 nM were applied.

PCR was performed in 96-well plates using a C1000™ Touch Thermal Cycler coupled with the CFX96™ Real-Time System (Bio-Rad, Munich, Germany) under the following conditions: 95 °C for 10 min; 45 cycles each of 95 °C for 15 s, 58 °C (system HV), 64 °C (system Wss2d01), 60 °C (systems Rmips02, Dtub03) for 1 min each; for the HV system, an elongation step at 72 °C for 10 s was added [21].

Optimization of real-time PCR

The Rmips02, Wss2d01 and Dtub03 systems were optimized with regard to the annealing temperature in temperature ranges of 58–70 °C. The HV system had already been optimized. The optimal primer concentration was determined by combining different primer concentrations (300–900 nM). Tests were performed with forward/reverse primer concentrations of 300 nM/300 nM, 300 nM/600 nM, 300 nM/900 nM, 600 nM/300 nM, 600 nM/600 nM, 600 nM/900 nM, 900 nM/300 nM, 900 nM/600 nM and 900 nM/900 nM in duplicates.

Droplet digital PCR

Reactions were performed in a total volume of 20 μ L using a 2.5 μ L template [c (DNA) = 10–20 ng/ μ L]. The master mix consisted of 10 μ L ddPCR Supermix for Probes (Bio-Rad, Munich, Germany), 0.5 μ mol/L of each primer, 0.2 μ mol/L of TaqMan® probe and *Aqua bide*st. In a twin.tec PCR Plate 96, semi-skirted, blue (Eppendorf, Hamburg, Germany), 39.5 μ L of master mix was introduced, 5.64 μ L of template was added and the plate was centrifuged at 480 g for 3 min. The mixture was homogenized by repeated pipetting, and was centrifuged again for 3 min at 480 g. Next, two aliquots, each holding 20 μ L, were pipetted into the wells of the DG8 Cartridges for the QX100/QX200 Droplet Generator (Bio-Rad, Munich, Germany). Then, 70 μ L of Droplet Generation Oil for Probes (Bio-Rad, Munich, Germany) was added to the cartridges. After droplets were generated in the Droplet Generator QX200™ (Bio-Rad, Munich, Germany), the reaction mixtures were pipetted into 96-well reaction plates, and the plates were sealed with Pierceable Foil Heat Seal (Bio-Rad, Munich, Germany) at 180 °C in the PX1 PCR Plate Sealer (Bio-Rad, Munich, Germany). Reaction was performed in the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Darmstadt, Germany) under the following conditions: initial denaturation step for 10 min

at 95 °C followed by 80 cycles of 30 s at 95 °C and 60 s at 64 °C for the system Wss2d01 and 60 °C for the systems Rmips02, Dtub03 and HV. Droplet stabilization was performed at 98 °C for 10 min and subsequently cooled to 4 °C. Droplet reading and copy-number calculation were done using the Droplet Reader QX200 and the QuantaSoft software version 1.7.4 (Bio-Rad, Munich, Germany). Fluorescence was measured in the FAM channel. All thresholds were manually adjusted based on background fluorescence.

Optimization of ddPCR

The systems Rmips02, Dtub03 and Wss2d01 were directly transferred to the ddPCR with regard to the temperature program. For the HV system, an annealing temperature of 60 °C was used for the ddPCR. To optimize the concentrations of primers and probes, 150 nM, 200 nM and 250 nM for the probes and 500 nM, 600 nM and 750 nM for the primers were investigated in duplicates. Data were analyzed using R software version 3.6.3 [34] and an R-script according to LIEVENS et al. [35]. The evaluation took into account the extent of rain, resolution and the amount of fluorescence.

Validation

The basis for validation in real-time PCR was CEN/TS 17,239–1:2019–06 [36]. The methodology was transferred accordingly for ddPCR. The values from the determination of the copy number by ddPCR were used to set the copy number.

Specificity tests in real-time PCR

The copy number of DNA reference samples was calculated using OD260 according to [36]. To check inclusivity, 11 reference samples were diluted to a calculated copy number of about 40 cp/μL using salmon sperm DNA [$c = 20$ ng/μL] as background DNA for durum wheat and rye, 10 for spelt, and 12 for common wheat. The determination was performed in duplicates with the respective specific system.

DNA extracts [$c = 500$ cp/μL] from the following animal and plant species were used in the exclusivity testing: soybean (*Glycine max*), maize (*Zea mays*), rice (*Oryza sativa*), emmer (*Triticum dicoccum*), triticale (*Triticale*), oats (*Avena sativa*), durum wheat (*Triticum durum*), common wheat (*Triticum aestivum*), rye (*Secale cereale*), millet (*Sorghum bicolor*), flax (*Linum usitatissimum*), kamut (*Triticum turgidum subsp. turanicum*), sugar beet (*Beta vulgaris*), spelt (*Triticum spelta*), barley (*Hordeum vulgare*), buckwheat (*Fagopyrum esculentum*), sunflower (*Helianthus annuus*), pumpkin (*Cucurbita pepo*), cattle (*Bos taurus*), pig (*Sus scrofa*), turkey (*Meleagris gallopavo*) and chicken (*Gallus gallus*). The ACT [37] (plant-specific DNA) and myneu [38]

(animal-specific DNA) systems were used to confirm the presence of amplifiable DNA.

Determination of the limit of detection and efficiency

DNA extracts of the reference samples were diluted to a final amount of 20 cp/5 μL, 10 cp/5 μL, 5 cp/5 μL, 2 cp/5 μL, 1 cp/5 μL and 0.1 cp/5 μL, respectively, using salmon sperm DNA [$c = 20$ ng/μL] as background DNA. Twelve PCR replicates were measured in real-time PCR per dilution level. To determine the efficiency of the systems, the concentrations 2500, 500, 100 and 50 copies per preparation were each measured in three PCR replicates. LOD_{95%} was determined using the POD tool [39] in the program R version 3.6.3. By plotting POD (probability of detection) against copy number in logarithmic order, the value for LOD_{95%} can be determined.

To determine the LOD_{95%} in ddPCR, DNA extracts of the reference samples were diluted to a final amount of 20 cp/2.5 μL, 10 cp/2.5 μL, 5 cp/2.5 μL, 2 cp/2.5 μL, 1 cp/2.5 μL and 0.1 cp/2.5 μL, respectively, using DNA from salmon sperm [$c = 20$ ng/μL] as background DNA. Twelve PCR replicates were measured per dilution level. Following the method of DOBNIK et al. [27], the measurements in a ddPCR assay were considered as an overall positive result only when the number of positive droplets was at least three.

Robustness of the systems in ddPCR

The Rmips02, Dtub03, HV and Wss2d01 systems in four replicates per factor combination were examined for robustness. Samples were diluted to 30–40 cp/μL with DNA from salmon sperm [$c = 20$ ng/μL]. Master mixes for the combinations were prepared and are shown in Table 2. Annealing temperatures from Table 1 were changed up and down by 1 °C. Results were analyzed by a one-factorial analysis of variance and Scheffé test [40] at $p = 0.05$ each using the IBM® SPSS® Statistics Subscription version 1.0.0.1327 software.

Results

Cereal-specific systems

Rmips02 (rye system)

Sequencing with primers Rmips01-fw and Rmips01-rv on three rye samples reveals two homologous sequences of R 01/1 and R 12/1 with a length of 134 bp. The sequence of R 14/1 shows a deletion at base 110 (see Fig. 1). In

Table 2 Factor combination for assessment of robustness according to [36]

Factor	Combination			
	1	2	3	4
Primer concentration	Unmodified	– 30%	Unmodified	– 30%
Probe concentration	Unmodified	– 30%	– 30%	Unmodified
Mastermix volume	16.5 µL (≈ 94.3%)	16.5 µL (≈ 94.3%)	18.5 µL (≈ 105.7%)	18.5 µL (≈ 105.7%)
Annealing temperature	+ 1 °C	– 1 °C	– 1 °C	+ 1 °C

Fig. 1 Sequences of samples R 01/1, R 12/1 and R 14/1. Yellow: Rmips01-fw, Rmips01-rv. Turquoise: Rmips02-fw, Rmips02-rv. Magenta: Rmips02-p. Green: Rmips01-p

preliminary tests, the Rmips01 system showed poor performance with regard to fluorescence maximum in real-time PCR with different rye samples (data not shown). As shown in Electronic Supplementary Material (Supp. Table 4), the calculated melting temperatures of forward and reverse primer differ by 4 K, and between forward primer and probe, the difference is about 14 K. Due to this fact the performance of PCR could be affected. Hence, the Rmips01 system needed to be optimized. Based on the sequence data, the system Rmips02 was developed.

Dtub03 (durum wheat-specific system)

Sequence analysis of the β -tubulin gene (MG852148.1) from durum wheat showed an insertion of 9 bp, as well as an insertion of 21 bp, when compared to the sequence of common wheat; see alignment in Electronic Supplementary Material (Supp. Fig. 1). These were used for the primer and probe design with specificity for durum wheat. When tested in silico using BLAST [33], the primer–probe system Dtub03 was found to be the most suitable. Primer Dtub03-rv met the criteria for specificity, because there was no match with any other species of genus *Triticeae*. Sequencing with primers Dtub05-fw and Dtub03-rv resulted in complete sequence alignment of all samples (DU 01/1, DU 08/1, DU 18/1, TRI 15/1, TRI 17/1 and TRI 19,236). The alignment of the sequences is shown in Fig. 2.

Wss2d01 (common wheat- and spelt-specific system)

The Wss2d01 [19] system is also specific for the D genome of spelt, which was confirmed by sequencing (see Fig. 3). Spelt and common wheat could be specifically detected alongside the other cereals using this optimized system.

γ -Gliadin gene (GAG56D) to differentiate between common wheat and spelt

The assumed spelt-specific system Sglia01, developed in this work, targets a 9-bp deletion in spelt varieties compared to common wheat varieties. Preliminary tests with the Sglia01 system in real-time PCR showed that the common wheat varieties, TRI 03/1, TRI 04/1, TRI 05/1, TRI 17/1, and TRI 18/2 showed false-positive results for this in the measurement. Furthermore, the spelt variety, SP 17/1, was negative with Sglia01. Results from sequencing seven spelt varieties and ten common wheat varieties confirmed that the aforementioned common wheat varieties do not have a 9-bp insertion in the γ -gliadin gene but that the spelt variety SP 17/1 does indeed have this insertion in this gene, while TRI 03/1, TRI 04/1, TRI 05/1, TRI 17/1, and TRI 18/2 lack this insertion (see Fig. 4). The genetic variability of the 9-bp insertion between spelt and common wheat makes this target unsuitable for differentiation between the two species.

MG852124.1 TCTCCTTTCCCTTATAAA--TATGAAAGGTACGCCCATAAATAGTGTTGATGGTTGTGAATT
GU017484.1 TCTCCTTAATATGTAATGTGC-----ACCATATTAGTATTGTGATGGTTGTGAACT
MG852122.1 TCTCCTTTCCCTTATATAAATGAAATGTATGCCATAAATAGTGTGA-
MG852123.1 TCTCCTTTCCCTTGAATAT-----TTGTGATGGTTGTGAAT-
DU 01/1 -----AGTGTGTGAGATGGTTGTGAAT-
DU 08/1 -----AGTGTGTGAGATGGTTGTGAAT-
DU 18/1 -----AGTGTGTGAGATGGTTGTGAAT-
TRI 15/1 -----AGTGTGTGAGATGGTTGTGAAT-
TRI 17/1 -----AGTGTGTGAGATGGTTGTGAAT-
TRI 19236 -----AGTGTGTGAGATGGTTGTGAAT-
MG852148.1 TCTCCTTTCCCTTGTCAAT--GAAATGTACGCCATAAATAGTGTTGAGATGGTTGTGAAT-

MG852124.1 CTGGAAGAGGGCCTGCCAGTCGAACCTTTGGAATATTTACATGTCCGGCACTCAATTATA
GU017484.1 CTGGAAGAGGGCCTGCCACTTGTACCTTTTGAATATTTACATGTCCAGCACTCATTTAGA
MG852122.1 -----TGGTTGTGAATATTTACATGTATGGGACTCATTTAAT
MG852123.1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
DU 01/1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
DU 08/1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
DU 18/1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
TRI 15/1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
TRI 17/1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
TRI 19236 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
MG852148.1 -----TCTGGAACCTTTGGAATATTTACATGTCCGGCACTCATTTAAT
** ***** *

MG852124.1 CATAATATC-----AGATTGTCCGGCCCACCAAATAAG
GU017484.1 TATCCATTATATCAGGGTGCAGGCTATATATCCTTTAGATTGTTGGCCCCACCAAATAAG
MG852122.1 TATACATTATATCAGGGTGCAGCGTATATATCCTTCAGATTGTTGGCCCCACCAAATAAT
MG852123.1 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
DU 01/1 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
DU 08/1 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
DU 18/1 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
TRI 15/1 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
TRI 17/1 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
TRI 19236 TATACATTATATCAGGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
MG852148.1 TATACATTATATCATGGTGCATTCTATATATCCTTCTGATTGTTGGCCCCACCAAATAAG
** * TAGTGTGAGATAGTCTAGTG

MG852124.1 TAGCATAAGAATCTTTTTTATCAG-----TGCAAATATTCTTGTGAGATAGTCTAGTG
GU017484.1 TACCATAACTACCTTTTATCAGTGTAAATAACTAAATATTCTGTGAGGTAGTCTAGTG
MG852122.1 TAGCATA--ACAGCCCTTTTATCAG-----CATAAATATTCTTGTGAGGTAGTCTAGTG
MG852123.1 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
DU 01/1 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
DU 08/1 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
DU 18/1 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
TRI 15/1 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
TRI 17/1 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
TRI 19236 TAGCATAAGAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
MG852148.1 TAGCATAAGAAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG
TAGCATAAGAAATCTTTTTTATCAG-----TGTAATATTCTTGTGAGGTAGTCTAGTG

MG852124.1 GTCGACAATAAATT--GTATAATAATGCA-----ATCCACCGATTGAAGATAGTGTC
GU017484.1 ATCAAAAAGGATT--GTATAATGATGC-----AATCCACCATCAAGACAGTGTT
MG852122.1 GTTGACAATAAATT--GTATAATAATGCAA-----TCCACCATCAAGATAGTGTC
MG852123.1 GTTGACAATAAATTGTATAATAATGCAA-----TCCACCATCAAGATAGTGTT
DU 01/1 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
DU 08/1 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
DU 18/1 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
TRI 15/1 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
TRI 17/1 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
TRI 19236 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
MG852148.1 GTTGACAATAAATTGTATAATAATGCAATCCATGCAATCCACCATCAAGATAGTGTT
* ** *****

MG852124.1 CTTATATTGTATTCTGCTTAAGTGCTTAGCAAGTCTGCAAGTGTACTCAATTGCTGTATT
GU017484.1 ----CTTATA----TTGTATTCTGTTACAGCAAGTCTTCAAGTGTACTGAATTGCTTTATT
MG852122.1 ----CTTATATTGACTATTCTGCTTAGCAAGTCTGCAAGTGTACTGAATTGCTGTATT
MG852123.1 ----CTTAAT---TTGTATTCTGCTTAGCAAGTCTGCAAGTGTACTCAATTGATGTATT
DU 01/1 ----CTTAAT---TTGTATTCTGCT-----
DU 08/1 ----CTTAAT---TTGTATTCTGCT-----
DU 18/1 ----CTTAAT---TTGTATTCTGCT-----
TRI 15/1 ----CTTAAT---TTGTATTCTGCT-----
TRI 17/1 ----CTTAAT---TTGTATTCTGCT-----
TRI 19236 ----CTTAAT---TTGTATTCTGCT-----
MG852148.1 ----CTTAAT---TTGTATTCTGCTTAGCAAGTCTGCAAGTGTACTCAATTGATGTATT
* * * *****

Fig. 3 Sequencing results from wSSII-D gene with primers Wss-2d01fw and Wss2d01rv (yellow). Probe Wss2d01p in green. Alignment of the sequenced samples for common wheat (TRI 03/1,

TRI 15/1, TRI 20/2), spelt (SP 03/1, SP 13/1, SP 18/1), *Triticum aestivum* wSSII-B gene (AB201446.1) and *Triticum aestivum* wSSII-A gene (AB201445.1)

For the Rmips02 and Dtub03 system, 60 °C was selected as an optimum annealing and elongation temperature, because maximum fluorescence is reached at almost 60 °C and it is more feasible to work at the same temperature with both systems. Dtub03 amplified kamut and durum wheat with equal specificity. For the system Wss2d01, 64 °C was chosen to avoid nonspecific reactions with durum, kamut, emmer and triticale.

the level of background fluorescence. Rain is the number of droplets between the fluorescence level of positive and the background level. For the systems Rmips02, HV, Dtub03 and Wss2d01, the resolution was above the suggested minimum value of 2.5 [35]; the rain was always below 1%. With higher primer concentrations, the intensities of the fluorescence signals also increased, while the probe concentration did not affect the signal intensity as much. The optimal combination for the Rmips02 system was 500 nM for the primers and 200 nM for the probe (resolution: 9.03; rain: 0.16%; see Fig. 5). For the system Wss2d01, an optimal combination of 600 nM for the primers and 150 nM for the probe was chosen, and for the HV and Dtub03, 600 nM for the primers and 200 nM for the probe were chosen as optimum.

For ddPCR, resolution and rain are relevant parameters. In this context, resolution describes the separation between positive and negative droplets. Resolution depends very much on the fluorescence signal of positive droplets and

Figure 6 presents the results of ddPCR optimization of reference samples at three different dilutions. The droplets were observed in only one fluorescence region in the samples. In the samples with the lowest dilution, the rain appears stronger, because there are significantly more positive droplets, increasing the probability of droplets with intermediate fluorescence. Nevertheless, the relative amount of rain in

relation to the number of droplets is not increased. All optimization data can be found in the Supplementary Material (Supp. Tables 9, 10).

Validation

Specificity tests in real-time PCR

The results of the inclusivity tests showed positive results for the tested systems Rmips02, Dtub03 and Wss2d01, in the reference samples with Cq values ranging from 29.16 to 33.06. For complete results, see the Electronic Supplementary Material (Supp. Tables 11, 12, 13).

The Wss2d01 system gave positive results in the exclusivity tests for spelt and common wheat samples. The Rmips02 system amplified only the rye and triticale samples. Dtub03 also amplified two of 13 common wheat samples (TRI 15/1, TRI 17/1) in addition to durum and kamut samples. Further examination of *Schwäbische-Dickkopf Landweizen* (TRI 15/1) and *LIAO CHUEN NO.5* (TRI 17/1) revealed sequence homology of these common wheat cultivars in the β -tubulin gene. The results of the exclusivity tests are presented in the Electronic Supplementary Material (Supp. Table 14).

Determination of the limit of detection

The LOD_{95%} in real-time PCR (with 95% confidence interval in brackets) in copies per reaction is 4.07 (2.59; 6.35) for Dtub03; 3.17 (1.99; 5.02) for HV; 2.43 (1.49; 3.90) for Rmips02; and 3.08 (1.91; 4.88) for Wss2d01. These data follow the Poisson distribution. Considering that, in case every target sequence is successfully amplified, i.e. copied without any error, the ideal LOD_{95%} is 2.996 copies per reaction, the values obtained are thus close to the best detection limit per reaction achievable [41].

The PCR efficiency of the individual systems could be determined through the calibration series included in real-time PCR. The efficiency can be considered "optimal" if it is 100% $\pm$ 10%, and the coefficient of determination (R^2) is at least 0.98 [36, 42]. All these criteria could be fulfilled for the systems Dtub03 (E = 100.0%), Rmips02 (E = 93.1%), HV (E = 92.9%) and Wss2d01 (E = 105.8%).

The LOD_{95%} determined in ddPCR (with 95% confidence interval in brackets) in copies per reaction are 9.89 (6.55; 14.84) for Dtub03; 9.48 (6.28; 14.24) for HV; 9.07 (6.00; 13.65) for Rmips02; and 13.26 (8.80; 19.74) for Wss2d01. According to DOBNIK et al. [27], test results by ddPCR are considered positive when three droplets give a positive fluorescence signal. Using this minimum value for a positive reaction, a systematic error can be prevented and accidental false-positive results can be excluded; however, this strict requirement leads to higher value for LOD_{95%} (see Fig. 7). The best achievable values for LOD_{95%} are the number of

target molecules that are required to have at least three copies per reaction with a probability of 0.05. This number depends on the efficiency of ddPCR assay and on the Poisson statistics as follows:

The probabilities of positive results in digital PCR results follow the Poisson distribution characterized by the parameter λ .

$$p(x) = \frac{\lambda^x e^{-\lambda}}{x!} \quad (1)$$

The probability p for at least three positive droplets [27], i.e., at least three target molecules per reaction, shall be 0.95.

$$p(\geq 3) = 0.95 \quad (2)$$

The parameter λ represents in this way the number of target copies per reaction necessary to have at least three copies with the given probability of 0.95.

$$0.95 = \sum_{x \geq 3} p(x) \quad (3)$$

This means that the probabilities for positive results derived from nominal 0, 1, or 2 copies are 0.05 or less. The reversion of this interrelationship is represented by the following equations.

$$0.05 = p(0) + p(1) + p(2) \quad (4)$$

$$0.05 = \frac{\lambda^0 e^{-\lambda}}{0!} + \frac{\lambda^1 e^{-\lambda}}{1!} + \frac{\lambda^2 e^{-\lambda}}{2!} \quad (5)$$

$$0.05 = e^{-\lambda} + \lambda e^{-\lambda} + \frac{\lambda^2}{2} e^{-\lambda} \quad (6)$$

The solution is found by iteration resulting in $\lambda = 6.2958$. This represents the best LOD_{95%} achievable. As a result, the values obtained for LOD_{95%} in ddPCR were higher than those in real-time PCR, where one copy of target molecules shall end up in the reaction with a probability of 0.95. Further data and the figures for the determination of the detection limit are presented in the Electronic Supplementary Material (Supp. Tables 15, 16 and Supp. Figs. 2, 3).

Robustness in ddPCR

Using a factorial analysis of variance ($p = 0.05$), followed by a Scheffé test ($p = 0.05$), a significant ($p < 0.05$) difference was found between combinations 2 and 3 in the Rmips02 system. Combinations 1 and 2 tended to have higher copy numbers (not significant). In combination 2, the highest copy number was determined in all systems, and in combination 3 (except Wss2d01 in combination 4), the lowest copy number was determined. Dtub03 had the smallest variation among

```

AF144104.1      GCAGCCATCCATACCATCATAACATTCATCATCATGCAGCAAGAACAACAAGAACAACAA
AF120267.1      GCAGCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
TRI18/2         ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
TRI17/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
TRI05/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
TRI04/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
TRI03/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP18/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP15/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP13/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP09/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP08/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP05/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAG-----CAAGAACAACAA
SP17/1          ---GCCATCCATACCGTCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAA
TRI01/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAA
TRI16/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAA
TRI19/2         ---GCCATCCATACCGTCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAA
TRI02/1         ---GCCATCCATACCGTCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAA
TRI20/2         ---GCCATCCATACCGTCATACATTCCATCATCATGCAGCAAGAACAACAAGAACAACAA
                *****
AF144104.1      CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
AF120267.1      CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI18/2         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI17/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI05/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI04/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI03/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP18/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP15/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP13/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP09/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP08/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP05/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
SP17/1          CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI01/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI16/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI19/2         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI02/1         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
TRI20/2         CAAGGCATGCATATCCTGCTGCCACTATATCAGCAGCAACAGGTGGGTCAAGGTACTCTC
                *****
AF144104.1      GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
AF120267.1      GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI18/2         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI17/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI05/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI04/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI03/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP18/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP15/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP13/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP09/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP08/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP05/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
SP17/1          GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI01/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI16/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI19/2         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGCTCAATTGGAGGCGATCAGGTC
TRI02/1         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAGNTCAATTGGAGGCGATCAGGTC
TRI20/2         GTCCAGGGCCAGGGCATCATCCAACCCCAACAACCAG-----
                *****

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Fig. 4 Sequencing results with primers Sglia01-fw and Sglia03-rv (binding sites marked in yellow) and alignment of the gene segment in the γ -gliadin gene of the samples for common wheat (TRI 01/1 to TRI 20/2) and spelt (SP 05/1 to SP 18/1), *Triticum aestivum* gamma-gliadin (GAG56D) gene (AF144104.1) and *Triticum spelta* gamma-gliadin (GAG56D) gene (AF120267.1)

combinations. However, positive results were obtained in all combinations and all amplicons. Therefore, all systems were sufficiently robust with respect to the factor combinations tested (primer and probe concentrations of -30%, deviating master mix volumes of 94.3% and 105.7%, temperature differences of ± 1 °C). The statistical calculations are provided in the Electronic Supplementary Material (Supp. Table 17–28).

Analysis of flour and semolina mixtures

From the values calculated for the cereal-specific systems for rye and common wheat, the proportions of flour and semolina of the respective cereal species in the flour and semolina mixtures were calculated according to Eq. 7. For the cereal type G1 or G2, k stands for the measured copy number, and C represents the C-value. The equation establishes a correlation between haploid genome weight and copy number of each cereal species in the mixture.

$$G1 \text{ (in \%)} = \frac{k_{G1} \times C_{G1}}{(k_{G1} \times C_{G1}) + (k_{G2} \times C_{G2})} \times 100 \quad (7)$$

Equation 7 Calculation of the proportion in % (w/w) of grain type G1 in a binary mix of grain 1 (G1) and grain 2 (G2). The calculation is based on the number of copies (k_{G1} & k_{G2}) determined for the respective grain type and the corresponding haploid genome weight (C_{G1} & C_{G2}).

Flour mixtures

For the blends of whole-grain flours (denoted RW x/y, where x and y represent the proportions of rye and wheat), Eq. 7 approximated the ratio of the weighed mass fractions of the two flours. For the 50/50 blend, the mass fraction was 52.8% for rye and 47.2% for common wheat. In the 60/40 and 90/10 blends, the determined flour proportions were 62.6% and 88.8% for rye and 37.4% and 11.2% for common wheat, respectively, which approximately corresponds to the mass proportion of the original initial weight (see Fig. 8a).

In contrast to the proportions of flour of the cereal types in the mixture of wholemeal flours, those in the mixtures of flours with different degrees of grinding (RoWe x/y) deviated from the weighed-in proportions. The determined common wheat percentage was too low in absolute terms by 15.1% (in RoWe 50/50), 14.0% (in RoWe 60/40) and

3.8% (in RoWe 90/10). The values determined by ddPCR led to a higher rye content in these samples than was actually weighed in (see Fig. 8a). The copy numbers, shown in Table 3, were determined for the respective cereal species by means of ddPCR. For further data, see Electronic Supplementary Material (Supp. Table 29).

Mixtures of common wheat and durum wheat semolina

With determined proportions of 99.05% durum and 0.95% common wheat semolina, the ratio of this 99/1 mixture was very close to the weighed-in proportion of 99% and 1%, respectively. In the mixture with 5% common wheat, only about half of the weighed-in proportion (2.84%) was determined by this ddPCR method (see Table 4). From the trend analysis, the proportion of durum wheat determined by ddPCR is significantly higher than the actual proportion at each point (see Fig. 8b). The data on the mixtures are given in the Electronic Supplementary Material (Supp. Table 30).

Discussion

Development and optimization of cereal-specific systems

Rye (*Secale cereale*)

PEGELS et al. [17] developed the rye system, which was based on the single-copy MIPS gene and showed good specificity on reference samples. For further optimization, the gene segment was sequenced on three reference samples and was used to develop the Rmips02 system. The Rmips02 system gave positive results in specificity tests for the reference sample of triticale, since triticale is a hybrid of common wheat and rye or durum wheat and rye by allopolyploidization [8].

Common wheat (*Triticum aestivum*)

MATSUOKA et al. [19] developed a system, which targets the Starch synthase II gene (wSSII-D) on chromosome 7D [19, 25] of hexaploid common wheat. Genome D is the target genome, which does not occur in the tetraploid durum wheat genome. Thus, the system Wss2d01 is suitable for distinguishing common wheat from durum wheat as well as the other cereal species tested. Spelt (*Triticum spelta*), as a closely related species of common wheat, showed a homologous gene sequence in the tested samples. The system Wss2d01 can therefore detect spelt as well as common wheat.

Fig. 5 Optimization of the primer and probe concentration in ddPCR for *Rmips02*. Probe-/primer concentration (in nM): A1&A2 (150/500), B1&B2 (150/600), C1&C2 (150/750), D1&D2 (200/500), E1&E2 (200/600), F1&F2 (200/750), G1&G2 (250/500), H1&H2 (250/600)

Durum wheat (*Triticum durum*)

Sequencing demonstrated homology between the sequences of one kamut sample and two common wheat varieties (*Schwäbischen Dickkopf-Landweizen* and *LIAO CHUEN NO.5*) in the Dtub03 system. Likewise, homology was expected between kamut and durum wheat given their close relationship [6]. *Schwäbischer Dickkopf-Landweizen* is a rare regional common wheat variety with a cultivated area of only 1.2 hectares (2012) in the region of Swabia [43]. The common wheat cultivar *LIAO CHUEN NO.5* (*LIAO CHUN 5*) is a Chinese breeding variety [44] and a cross of *LIAOCHUN-1* and *FRONTANA* with a cultivated area of 67,000 ha from 1966 to the late 1970s [45]. To our knowledge, both varieties do not have any economic relevance.

Barley (*Hordeum vulgare*)

Zeltner et al. [21]. developed the barley system HV for real-time PCR, which provides specific results for barley (*Hordeum vulgare*) [21]. When transferred to ddPCR, the annealing and elongation temperature was optimized to 60 °C in regards to a potential multiplex ddPCR method.

Differentiation between common wheat and spelt

In previous publications, a 9-bp insertion in the γ -gliadin gene (GAG56D) of common wheat was used to distinguish common wheat from spelt [16, 22]. However, by sequencing the gene segment around the region of the insertion, no insertion was detected in five of ten common wheat

reference samples. One of seven spelt reference samples (SP 17/1) exhibited this insertion in the gene sequence, which was considered to be specific for common wheat. This demonstrated that this marker was unsuitable for distinguishing between the two cereal species as no reliable results could be obtained.

As 2nd putative molecular target, a 160-bp insert in the Wx-A1 (HQ625382) gene of spelt was tested by several primer pairs, PCR results however gave false-positive results in common wheat when assessed experimentally, see Electronic Supplementary Material (Supp. Table 4).

Spelt (*Triticum spelta* or *Triticum aestivum* subsp. *spelta*) and common wheat (*Triticum aestivum* or *Triticum aestivum* subsp. *aestivum*) are very closely related in evolutionary biology [7]. Analyses of the growing number of published sequences of *Triticum spelta* and *Triticum aestivum* from GenBank® did not provide a molecular basis for differentiating the two species by a PCR system targeting a single genetic sequence. Putative genetic species-specific genetic elements between both species differ remarkably between several breeding lines. Despite several approaches, a distinct molecular system could not be applied to distinguish spelt and common wheat reliably, see Electronic Supplementary Material (Supp. Table 4). In conclusion, it is not possible to distinguish both species reliably by one PCR system for the time being. Thus, additional research is needed. One possible approach to facilitate this could be a multifactorial analysis of single-nucleotide polymorphisms (SNP`s).

Evaluation of the flour mixtures

Equation 7, shown in the results, was used to calculate the proportions in a cereal type. This is achieved by assuming there is a 1:1 mixture of common wheat/rye dsDNA with a dsDNA concentration of 100 ng/μL each. The calculated copy numbers are about 11,364 cp/μL for the rye dsDNA (C-value 8.8 [46]) and about 5780 cp/μL for the common wheat dsDNA (C-value 17.3 [46]). Due to the lower haploid genome weight of rye dsDNA, there is a factor of 1.97 (ratio 17.3:8.8) more copies in the same reaction mixture per μL at the same dsDNA concentration. With this relationship, the flour fraction in the mixtures determined by ddPCR can be calculated using the aforementioned equation. There is a correlation between flour type and DNA content. The flour type is directly related to the degree of milling (proportion of flour/proportion of separated bran). Rye flour type 997 has a degree of milling of about 74–79% and common wheat flour type 550 about 65–72% [47]. A higher degree of milling of a flour in mixtures results in a higher DNA copy number in the extract and thus a higher determined proportion of a cereal species. Accordingly, the flour fractions determined by ddPCR in a flour mixture are only identical to the flour fractions actually weighed if the degrees of grinding of the

Fig. 6 Copy-number determination by results of optimized ddPCR systems. **a)** HV system with samples H 19/1 1:30 (B01, B02), H 19/1 1:150 (D01, D02) and H 19/1 1:750 (F01, F02). **b)** Dttub03 system with samples DU 06/1 1:50 (B01, B02), DU 06/1 1:250 (D01, D02) and DU 06/1 1:1250 (E01, E02). **c)** Rmips02 system with sam-

ples R 11/1 1:45 (C03, C04), R 11/1 1:225 (E03, E04) and R 11/1 1:1125 (F03, F04). **d)** Wss2d01 system with samples TRI 19/2 1:20 (A01, A02), TRI 19/2 1:100 (B01, B02), TRI 19/2 1:500 (C01, C02), SP 18/1 1:20 (D01, D02), SP 18/1 1:100 (E01, E02) and SP 18/1 1:500 (F01, F02)

flours are almost the same. Areas with higher cell density will have a greater amount of DNA similar to the differences between embryo and endosperm in *Zea mays* [48]. Knowledge of the types of flour is therefore important in making accurate assessments of flour blends in foods. Due to the higher price of rye flour, adulteration by stretching with common wheat flour is more common than doing so with rye.

Evaluation of semolina mixtures

The background for the analysis of these mixtures is Regulation (EU) No. 2016/1238, which stipulates a maximum foreign

grain content in durum wheat of 3%. By producing semolina mixtures, a common wheat content of 1%, 2%, 3% and 5% was simulated in the remaining proportion of durum wheat semolina. Thus, the proportion from common wheat semolina is significantly underestimated, and the proportion from durum wheat is overestimated. Since no degree of grinding is usually specified for semolina, a correlation is not applicable.

Applications of ddPCR in analysis of the cereal products

Compared to real-time PCR, results of ddPCR are not subject to the inherent errors of copy-number determination. The

Fig. 7 Comparison of $LOD_{95\%}$ for Rmips02. **a)** POD diagram for Rmips02 in real-time PCR. **b)** POD diagram for Rmips02 in ddPCR. $LOD_{95\%}$ shown with 95% confidence interval (CI). (X=nominal

DNA concentration; S=number of positive replicates; N=number of tested replicates; green line defines the absolute DNA copy number when POD is 0.95)

Fig. 8 a Analysis of the mass fractions in the flour mixes. Blue triangles: proportion of wholemeal rye flour ($y_1 = 0.8921x_1 + 0.086$; $R^2 = 0.9987$) – Blue circles: proportion of whole wheat flour ($y_2 = 0.8921x_2 + 0.022$; $R^2 = 0.9987$) – Red crosses: proportion of rye flour type 997 ($y_3 = 0.702x_3 + 0.3085$; $R^2 = 0.9902$) – Red diamonds: proportion of common wheat flour type 550 ($y_4 = 0.702x_4 - 0.0105$;

$R^2 = 0.9902$). **b** Trend analysis of the mass fraction of semolina. Green: proportion of durum wheat semolina ($y_5 = 0.4776x_5 + 0.5183$; $R^2 = 0.9999$). Violet: proportion of common wheat semolina ($y_6 = 0.4776x_6 + 0.0041$; $R^2 = 0.9999$). Trend values (marked with x) created with the "TREND" function in Microsoft EXCEL 2019

Table 3 Results of the ddPCR analysis of the mixtures of whole rye flour with whole wheat flour (RW x/y), as well as rye flour type 997 with wheat flour type 550 (RoWe x/y) in the mass ratios of 50% rye with 50% common wheat, 60% rye with 40% common wheat and 90% rye with 10% common wheat. All mixtures were prepared in duplicate. The determined percentage of flour was calculated with Equation 17 using the determined number of copies and the corresponding C-values

Sample	Calculated copies/ μ L in sample		Determined percentage of flour in % (w/w)			
	Rmips02	Wss2d01	Rye		Common wheat	
RW 50/50 1	47,832	22,176	52.3	52.85	47.7	47.15
RW 50/50 2	37,048	16,434	53.4		46.6	
RW 60/40 1	26,016	7776	63.0	62.60	37.0	37.40
RW 60/40 2	34,192	10,592	62.2		37.8	
RW 90/10 1	79,240	4942	89.1	88.80	10.9	11.20
RW 90/10 2	45,120	2992	88.5		11.5	
RoWe 50/50 1	47,200	12,240	66.2	65.10	33.8	34.90
RoWe 50/50 2	8888	2540	64.0		36.0	
RoWe 60/40 1	47,200	8040	74.9	74.05	25.1	25.95
RoWe 60/40 2	68,360	12,720	73.2		26.8	
RoWe 90/10 1	83,800	3120	93.2	93.75	6.8	6.25
RoWe 90/10 2	100,200	3060	94.3		5.7	

Table 4 Results of the ddPCR analysis of the mixtures of durum wheat semolina with common wheat semolina (HaWe x/y) in the set mass ratios of 99% durum wheat with 1% common wheat, 98% durum wheat with 2% common wheat, 97% durum wheat with 3%

Sample	Calculated copies/ μ L in sample		Determined percentage of semolina in % (w/w)			
	<i>Dtub03</i>	<i>Wss2d01</i>	Durum wheat		Common wheat	
HaWe 99/1 1	24,480	169	99.06	99.05	0.94	0.95
HaWe 99/1 2	19,600	138	99.05		0.95	
HaWe 98/2 1	24,600	234	98.72	98.66	1.28	1.34
HaWe 98/2 2	18,880	196	98.60		1.40	
HaWe 97/3 1	21,360	294	98.15	98.23	1.85	1.77
HaWe 97/3 2	24,600	312	98.30		1.70	
HaWe 95/5 1	19,760	397	97.33	97.16	2.67	2.84
HaWe 95/5 2	22,200	504	96.99		3.01	

common wheat and 95% durum wheat with 5% common wheat. All mixtures were prepared in duplicate. The determined percentage of semolina was calculated with Equation 71 using the determined number of copies and the corresponding C-values

assessment of validation parameters for real-time PCR applications requires an exact quantification of the DNA concentration of reference material by photometrical or fluorimetric DNA quantification methods. The same implies to quantitative real-time PCR applications when using standard curves. Digital PCR allows an absolute quantification resulting in a higher degree of reliability. In contrast to real-time PCR, quantification by ddPCR omitted the implementation of a standard curve for the evaluation of the results. Despite the higher complexity of ddPCR, finally, this saves resources, especially when applied to multiple targets in a single test sample. The differentiation between detection and quantification by several reactions is dispensable.

Droplet digital PCR allows reliable detection and absolute quantification of common wheat, durum wheat, rye and barley. The results of quantification depend on the DNA content of each of the ingredients depend on the DNA content after milling and further processing and are therefore subject to error. This needs to be taken into account by interpreting quantitative data. Within this limitation, quantitative results give indications of adulteration. These analytical results may need to be verified by other methods for legal consequences, this might not necessarily be analytical methods. Our results of flour and semolina mixtures, made of commonly available ingredients, indicate an underestimation of the “less valuable” ingredient common wheat. Based on data presented here, there is no risk of unfair legal consequences. We consider these results of one of several elements to discover less careful production or even fraudulent activities in the food chain.

Conclusion

A molecular method for the differentiation of the cereal species spelt (*Triticum spelta*) and common wheat (*Triticum aestivum*) could not be achieved within the scope of

this work. The developed systems *Wss2d01*, *Rmips02*, *Dtub03* and *HV* were developed for ddPCR and adapted with respect to temperature program and oligo concentrations. In addition, the methods were validated with respect to specificity (inclusivity and exclusivity), detection limit and robustness. *Dtub03* also yielded positive results for the common wheat cultivars, *Schwäbische-Dickkopf Landweizen* and *LIAOCHUN 5*. *Schwäbische-Dickkopf Landweizen* is only cultivated in the region of Swabia and therefore has only little economic relevance. *LIAO-CHUN 5*, as a Chinese variety from the 1970s, is also likely to have little economic relevance in the present.

In the flour and semolina mixtures analyzed, the corresponding cereal species (flour: rye and common wheat; semolina: durum and common wheat) were successfully detected by ddPCR. Application of the barley-specific system in such mixtures is also possible. Quantification of mixtures showed a correlation between the copy number determined by ddPCR and the haploid genome weight of the cereal species was found. Through this correlation, the proportion of cereal species mixtures can be directly calculated from the determined copy number. However, correct assessment of flour mixtures must always take into account the degree of grinding, which has a decisive influence on DNA copy numbers. For further research, more approaches for molecular differentiation of the closely related species spelt and common wheat are needed.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

Compliance with ethics requirement This article does not contain any studies with human or animal subjects.

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