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Original article

Traceability of the local cultivar 'Caaveiro' in flour mixtures used to produce Galician bread by simple sequence repeats and droplet digital polymerase chain reaction technology

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Summary The analysis of simple sequence repeats (SSRs) and of bulk ground samples by droplet digital polymerase chain reaction (ddPCR) was investigated as an alternative to individual kernel testing for assessing the presence of local cultivars in common wheat (*Triticum aestivum* L.) varietal blends. The recent Protected Geographical Indication (PGI) of 'Pan Galego' (Galician bread) requires that the flour comprise a minimum 25% local wheat cultivars. As a test for compliance with this minimum level, wheat flours were prepared by mixing commercial flours with 0%, 5%, 20%, 25% and 30% 'Caaveiro' and 100% 'Caaveiro' and 100% commercial flours were used as controls. A second analysis was performed with a second set of wheat flours with 5% and 25% 'Caaveiro'. These were mixed with two different commercial flours to assess the potential ability of five SSRs to identify the percentage of 'Caaveiro', constituting the first reference of the use of SSRs in the traceability of specific autochthonous cultivars in flour blends. ddPCR using the QX200 system platform was used to the targeted proportions across the simulated range with two out of five SSRs, indicating that they can be used in the traceability of 'Caaveiro' in mixed flours and breads.

Keywords autochthonous wheat, ddPCR, flour blends, microsatellites, traceability.

Introduction

In Spain, six million ha of cereals are cultivated producing 25.4 million t of cereal grain, of which 7.1 t are wheat (MAPA, 2020). Commercial flours used in the Galician are currently produced by mixing commercial wheat cultivars, depending on the year, with the Galician cultivar 'Caaveiro' to produce Galician bread (Câmara-Salim *et al.*, 2020). The cultivar 'Caaveiro' is a soft winter wheat, which imparts a distinct flavour and taste to bread. The recently created Protected Geographical Indication (PGI) Pan Galego (UE, 2019a) commits to the inclusion of a minimum of 25% Galician wheat. Nowadays, there is no way to certify that this minimum is accomplished by flour mills or bakeries in the production of Galician bread under the auspices of the PGI.

'Pan Galego'/'Pan Gallego' (PGI) (2019/C 243/03) (UE, 2019b) 'is a crusty bread (the hardness of the crust varying depending on the format) with a soft and springy crumb dotted with a great many irregular air pockets. It is made by artisan bakeries using common wheat flour, some of which is made from native Galician wheat varieties and ecotypes (those commonly known as "trigo país" (autochthonous wheat) or "trigo gallego" (Galician wheat)) grown in the Autonomous Community itself. The bread is made using a sourdough starter (making up at least 15% of the total flour weight) and a large amount of water (at least 75 L per 100 kg of flour), with long rising (minimum 3 h) and baking times. Ovens floored with stone or other refractory materials are used for baking.'

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'Processes are slow as a result of using sourdough starters for fermentation, resulting in a soft and springy crumb, lots of air pockets, an intense flavour and a touch of acidity.' (UE, 2019b) 'The use of flour made from local wheat ecotypes, mixed with the expert eye of Galicia's bakers – with flours from outside Galicia, is another typical feature of this bread that contributes to its specificity. The qualities conferred on 'Pan Gallego' by most of the local wheat ecotypes (native wheats) derive from their high protein content and extensibility, plus the fact that the gluten they contain is ideal for bread-making. However, as these are weak or medium-strong flours, local bakers have traditionally combined them in varying proportions with stronger, more elastic flours, usually imported from Castile. The use of these native flours also affects the colour of the crumb, making it darker than that found in other wheat breads, and, according to the artisan bakers, gives the bread more intense aromas and flavours. On this last point, it has been demonstrated that the bread's aromas derive from certain components of the flour, which is why using these wheats would affect its organoleptic characteristics.' (UE, 2019b).

Droplet digital polymerase chain reaction (ddPCR) is considered the next generation of PCR (Hindson *et al.*, 2011). It is performed by partitioning the reaction mixture into an emulsion of minuscule droplets and treating each one as an endpoint reaction. The proportion of PCR outcomes defines the absolute quantification of a specific sequence. ddPCR allows absolute quantitation of nucleic acids in a sample without the need for standards. It is less affected by inhibitors due to dilution by partitioning the reactions into thousands of nanodroplets and the insignificance of primer efficiencies. A single PCR mixture is divided into over 20 000 water-in-oil droplets as a base for PCR amplification of single template molecules. A DNA binding dye is included in the Super-Mix in order to differentiate positive droplets (amplification of the target molecule) from negative droplets (no target molecule present) by using automated droplet flow cytometry. Finally, the number of target molecules in the mixture is estimated from the positive and negative droplets by using Poisson statistics.

Primer-probe systems for real-time PCR and droplet digital PCR (ddPCR) have been used previously for the detection of cereal species (Schulze *et al.*, 2021). ddPCR was effective in detecting and quantifying species of rye and common wheat in mixtures, but the results depended on the DNA content, and the ploidy of cereal species and were also influenced by comminution.

ddPCR has been used to perform quantitative analysis of bulk samples rather than individual seeds in varietal blends of wheat (Perry & Lee, 2017). Resistance to orange wheat blossom midge *Sitodiplosis mosellana* (Géhin), a relevant insect pest in wheat, has been identified under the control of the single gene Sm1, which has been incorporated into several wheat varieties, such as 'Unity'. However, the breakdown of resistance of this single gene through evolution of virulence by the wheat midge was expected. Hence, an interspersed refuge strategy was implemented by mixing midge-tolerant wheat (MTW) seeds with a midge-susceptible cultivar in a 9:1 ratio. This blend was submitted to verification testing to ensure that the blend ratio remained within the acceptable range. Tests were performed on individual seeds, and up to 1000 seeds were tested per sample. ddPCR was used successfully to verify that the coefficients of variation had been achieved, as they were generally below 5%. In another study, a duplex chip digital PCR (cdPCR) assay was used to quantify the 3% of contamination by wheat in the pasta production chain, from raw materials to the final products based on durum wheat (*Triticum durum*) (Morcia *et al.*, 2020).

Although studies based on SSRs have been conducted to identify wheat cultivars (Ruiz *et al.*, 2012) and on ddPCR to quantify the presence of a specific cultivar or species in mixtures of seeds (Perry & Lee, 2017) or flours (Morcia *et al.*, 2020), there have been no previous studies on the use of SSRs to identify Galician cultivars or to quantify their presence in blends at the minimum 25% level required by the PGI 'Galician bread' and no studies on the use of ddPCR for the same purpose.

The objective of this study was to test eighteen simple sequence repeats (SSRs) for their usefulness in identifying wheat cultivars in the traceability of the autochthonous cultivar 'Caaveiro' in the flours used for the production of 'Galician bread' under the auspices of the PGI 'Pan Galego'/'Pan Gallego' and to test five out of the eighteen SSRs by ddPCR. This is the first study to identify and quantify the use of Galician wheats in the production of 'Galician bread' by using SSRs and ddPCR.

Material and methods

Materials

We evaluated two Galician cultivars, 'Caaveiro' and 'Callobre' one Galician ecotype 'Carral'; and fourteen commercial cultivars: 'Enebro', 'Nogal', 'Rebelde", 'Ovalo','Valbuena', 'Montecarlo', 'Algoritmo', 'Basilio', 'Tocayo', 'Atomo', 'Sensos', 'Acorazado', 'Alhambra', and 'Radia'. Additionally, we evaluated three commercial flours for bread, named type A ('Castilian strong force' used by Da Cunha group), B ('Carrefour' brand) and C ('Eroski bio' brand), all of them blends of commercial wheat cultivars.

First trial
flour with 100% local cultivar 'Caaveiro' and commercial flour (type A) with 0%, 5%, 20%, 25% and 30% of 'Caaveiro' flour.

Second trial
flour with 100% local cultivar 'Caaveiro', 95% commercial flour (type B) with 5% 'Caaveiro' flour, 75% commercial flour (type B) with 25% 'Caaveiro' flour, and 75% commercial flour (type C) with 25% 'Caaveiro' flour.

DNA extraction

A 0.5 g sample of flour or grain was used for DNA extraction with the E.Z.N.A.®Plant DNA Kit (OMEGA Bio-Tek Inc., Norcross, GA, USA) and DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany).

SSRs

DNA integrity and quality were assessed by gel electrophoresis and visualisation of the previously stained samples. DNA samples were quantified using the Invitrogen Qubit 4 Fluorometer with the Qubit dsDNA HS (High Sensitivity) Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Five out of eighteen SSRs adapted from durum wheat (*Triticum turgidum* L.) (Ruiz *et al*., 2012) were selected from a previous analysis (unpublished) with soft wheat samples to identify specific markers for autochthonous wheat cultivars and for ddPCR (Table 1). A combination of high Polymorphism Information Content (PIC) values, a high number of alleles and location in different Linkage Group (LG) was sought.

PCR

Polymerase chain reaction amplifications were performed in a final volume of 20 μL containing 12 mM PCR buffer pH 8.3, 2.0 mM MgCl2, 0.31 mM of each nucleotide (dNTPs), 1 μM of the primer Forward, 1 μM from the primer Reverse, 1 μd of Taq DNA polymerase and 50 ng of DNA, completed with distilled water up to 20 μL.

With these microsatellite primers, the samples were amplified in a thermocycler (PTC-100 from M.J. Research, INC or GeneAmp PCR System 2700) based on the protocol of Reija (Reija Abelairas, 2017).

Polymerase chain reaction amplification was performed following the thermocycling program described below, taking into account that the 'annealing' temperature selected was optimal for the SSRs in each of the multiplexes: 94 °C for 2 min, followed by thirty cycles of 94 °C for 35 s, the annealing temperature for 1 min 30 s, 72 °C for 1 min 30 s and a final extension of 5 min at 72 °C. Multiplexes, as expected after set-up, showed amplification products for all SSRs (Table 1).

Multiplexes, as expected after set-up, showed amplification products for all SSRs (Table 1).

ddPCR

Previously designed primers were used to perform ddPCR experiments with EvaGreen Dye (Table 1).

Each reaction contained 1× mix (QX200 EvaGreen Digital PCR Supermix, Bio-Rad Laboratories, Hercules, CA, U.S.A.), 200 nM of each forward and reverse primer solution, and 2.03 μL of DNA template, resulting in a final volume of 20.3 μL. Droplets were generated with the QX200 droplet generator (Bio-Rad Laboratories), and PCR was performed in a C1000 touch thermal cycler.

A thermal-gradient PCR experiment was conducted to establish the optimal annealing temperature for the primers. The temperatures tested were 55.0, 56.4, 58.5, 60.9, 62.9, 64.3, and 65 °C. The negative control (NDT), water, was run at 54.2 °C. PCR thermal cycling conditions were as follows: initial denaturation stage of 95 °C for 10 min, followed by forty cycles of denaturation at 94 °C for 30 s, optimal annealing temperature for 60 s, and a final incubation of 10 min at 98 °C. PCR plates were transferred into a QX200 droplet reader (Bio-Rad), and readings were analysed using QuantaSoft software (Bio-Rad). The annealing temperature for PCR amplification was set at 59 °C for the five assays (Fig. 1).

First trial
Serial dilutions (1:2; D1 and D2) (Table 2) of the stock DNA were prepared for quantification purposes by ddPCR for each of the six samples including 0, 5, 20, 25, 30, and 100% autochthonous 'Caaveiro' flour with an industrial flour used in Galicia for bread (Type A). A 2.03 μL volume of each dilution for each sample was added to each one of the five assays (Fig. 2).

Second trial
The same amplification conditions were employed as described above. Four new samples (S7, 100% local cultivar 'Caaveiro'; S8, 95% commercial flour type B with 5% 'Caaveiro' flour; S9, 75% commercial flour type B with 25% 'Caaveiro' flour, and S10, 75% commercial flour type C with 25% 'Caaveiro' flour) were diluted to 20 ng/μL (D3 for Xgwm0312 and Xgwm0156), 10 ng/μL (D4 for Xgwm0099 and Xgwm0332) and 5 ng/μL (D5 for Xgwm0570) and the working concentration was chosen from previous trial criteria. Reactions were performed in duplicate for each of the five assays (Fig. 3).

Table 1 SSRs evaluated in this study in Galician flours and bread. Adapted from Ruiz *et al.* (2012)

SSRs	LG	Multiplex	Fluoroc hrome	Number of alleles (unique alleles)	PIC	Primer forward	Primer reverse	Repetition motive	T° of annealing	Opata wheat (bp)	Synthetic wheat (bp)
Xgwm0186	5A	1	VIC	28 (15)	0.77	GCA GAG CCT GGT TCA AAA AG	CGC CTC TAG CGA GAG CTA TG 5′	(GA)26	60°	132	106
Xgwm0234	5B	1	6-FAM	19 (9)	0.75	GAG TCC TGA TGT GAA GCT GTT G	CTC ATT GGG GTG TGT ACG TG	(CT)16(CA)20	55°	250	229
Xgwm0312*	2A	1	6-FAM	26 (4)	0.85	ATC GCA TGA TGC ACG TAG AG	ACA TGC ATG CCT ACC TAA TGG	(GA)37	60°	216	219
Xgwm0577	7B	1	NED	41 (15)	0.77	ATG GCA TAA TTT GGT GAA ATT G	TGT TTC AAG CCC AAC TTC TAT T	(CA)14(TA)6	55°	164	155
Xgwm0099*	1A	2	6-FAM	11 (3)	0.94	AAG ATG GAC GTA TGC ATC ACA	GCC ATA TTT GAT GAC GCA TA	(CA)21	60°	117	120
Xgwm0148	2B	2	NED	12 (4)	0.8	GTG AGG CAG CAA GAG AGA AA	CAA AGC TTG ACT CAG ACC AAA	(CA)22	60°	165	167
Xgwm0155	3A	2	VIC	16 (3)	0.86	CAA TCA TTT CCC CCT CCC	AAT CAT TGG AAA TCC ATA TGC C	(CT)19	60°	143	127
Xgwm0156*	5A	2	VIC	20 (4)	0.91	CCA ACC GTG CTA TTA GTC ATT C	CAA TGC AGG CCC TCC TAA C	(GT)14	60°	300	279
Xgwm0332*	7A	2	6-FAM	20 (4)	0.86	AGC CAG CAA GTC ACC AAA AC	AGT GCT GGA AAG AGT AGT GAA GC	(GA)36	60°	290	211
Xgwm0570*	6A	2	PET	15 (3)	0.9	TCG CCT TTT ACA GTC GGC	ATG GGT AGC TGA GAG CCA AA	(CT)14(GT)18	60°	149	143
Xgwm0060	7A	3	NED	19 (5)	0.8	TGT CCT ACA CGG ACC ACG T	GCA TTG ACA GAT GCA CAC G	(CA)30	60°	190	224
Xgwm0088	6B	3	6-FAM	17 (4)	0.72	CAC TAC AAC TAT GCG CTC GC	TCC ATT GGC TTC TCT CTC AA	(GT)18TT(GA)4	60°	162	–
Xgwm0389	3B	3	VIC	14 (3)	0.75	ATC ATG TCG ATC TCC TTG ACG	TGC CAT GCA CAT TAG CAG AT	(CT)14(GT)16	60°	117	128
Xgwm0513	4B	3	PET	4 (0)	0.79	ATC CGT AGC ACC TAC TGG TCA	GGT CTG TTC ATG CCA CAT TG	(CA)12	60°	152	146
BARC155	5A	4	6-FAM	11 (4)	0.67	GCG AGT ATT GAC GTC TTA TTT TTG AA	GCG TCA TGA ATT CTA ACA ATG TGC ATA	(TAA)10	50°	182	
BARC80	1B	4	NED	17 (6)	0.88	GC GAA TTA GCA TCT GCA TCT GTT TGA G	CGG TCA ACC AAC TAC TGC ACA AC		51	112	103
WMC468	4A	4	VIC	5 (1)	0.5	AGC TGG GTT AAT AAC AGA GGA T	CAC ATA ACT GTC CAC TCC TTT C		51°		
Xgwm0002	3A	4	PET	4 (0)	0.36	CTG CAA GCC TGT GAT CAA CT	CAT TCT CAA ATG ATC GAA CA	(CA)18	50°	128	130

*SSRs selected from previous analysis (unpublished) with soft wheat samples to identify specific markers for autochthonous wheat cultivars for ddPCR.

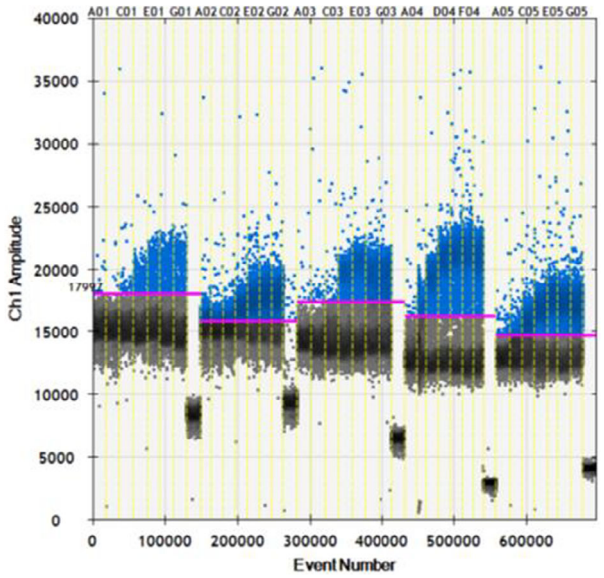

Figure 1 Temperature amplification gradient with the sample S1 and the five SSRs (from left to the right, Xgwm0312, Xgwm0099, Xgwm0156, Xgwm0332, Xgwm0570) from left to right: 65; 64.3; 62.9; 58.5; 56.4; 55 and 54.2 °C.

Table 2 Serial dilutions (1:2; named D1 and D2) of the stock DNA for each of the six samples prepared for quantification purposes by ddPCR

Sample	% of 'Caaveiro' flour mixed with commercial flour (type A)	Dilutions	ng/μL
S1	0	D1	37.50
	0	D2	9.38
S2	5	D1	20.20
	5	D2	5.05
S3	20	D1	24.30
	20	D2	6.08
S4	25	D1	17.60
	25	D2	4.40
S5	30	D1	27.10
	30	D2	6.78
S6	100	D1	25.20
	100	D2	6.30

Results

SSRs and alleles

Capacity to distinguish of flours and their percentages

Selected SSRs were able to distinguish the autochthonous wheat 'Caaveiro' from other cultivars, showing specific alleles for Xgwm0312, Xgwm0332, Xgwm0099, Xgwm0570, Xgwm0060 and WMC468 (Table 3). Moreover, commercial flours used for bread also showed specific profiles, a combination of the cultivars mixed every year (Table 3).

Once the monovarietal flours ('Caaveiro') and their mixtures were subject to PCR amplification, it was found that they had not undergone changes in DNA quality due to milling (Figure S1), and the PCR products obtained were of very good quality; therefore, PCR is a very useful tool for traceability studies and the detection of fraud in flours.

On the other hand, not all SSRs were useful for this purpose (Table 3), since some of them had common alleles in monovarietal flours and, therefore, flour mixtures contained matching alleles. An example of this was Xgwm0148, for which 'Caaveiro' flour presented allele 144, and type A flour presented alleles 144 and 160. Therefore, mixtures of both flours presented alleles 144 and 160, but this result does not discriminate a pure type A flour from one mixed with 'Caaveiro', since both would present the common allele 144 (Figure S2). Therefore, the microsatellites Xgwm0148, Xgwm0155, Xgwm0186, Xgwm0234, Xgwm0088, Xgwm0513, Xgwm0389, Xgwm0577, BARC155, BARC80 and Xgwm0002 were not useful for this purpose (Table 3).

On the other hand, the SSRs Wgwm0156, Wgwm0312, Wgwm0332, Wgwm0099, Wgwm0570, Xgwm0060 and WMC468 were useful in this differentiation (Table 3). In the case of the microsatellites Wgwm0312, Wgwm0099 and Xgwm0060, the differentiation capacity was larger (indicated in Table 3 as '++'), because the alleles are not repeated in monovarietal flours.

For example, in the case of locus Xgwm0312 (Figure S3), 'Caaveiro' presented alleles 250 and 252, while type A flour showed alleles 192, 194, 208, 214, 222 and 230; hence, alleles 250 and 252 were not repeated. Thus, when the two mixtures are combined, it should be noted that the alleles of both varieties appear in proportion to the amount of each flour type and do not overlap.

However, Wgwm0156, Wgwm0332, WMC468 and Wgwm0570 (Table 3, indicated with '+', Figure S4) predicted the presence or absence of flours depending on their origin, but the degree of precision was lower, since there are one or more alleles in common between the monovarietal 'Caaveiro' and type A flours. This case is depicted in Figure S4, where the 190 allele is repeated in both the 'Caaveiro' and type A flours. Consequently, this allele has a low discrimination capacity, as it will always appear, and its peak height will not indicate the percentage of each variety.

Once the percentage composition of all the mixtures had been analysed, it was possible to quantify the proportion of wheat in the blend with a minimum level of detection of 5% (Figure S5) by the comparison of the discriminant allelic profiles according to

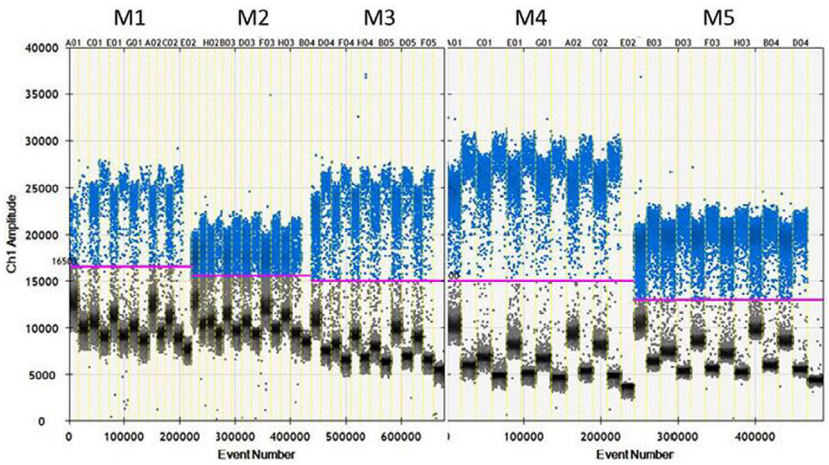

Figure 2 From left to the right, dilutions D1 and D2 for each sample (S1, 0% 'Caaveiro', to S6, 100% Caaveiro, plus a negative control, water) for each SSR (M1–M5). Red lines show the manually set threshold for each assay.

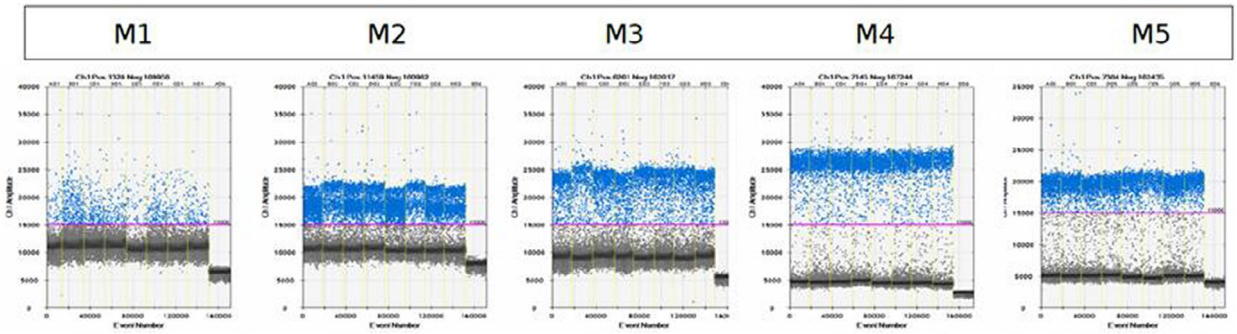

Figure 3 From left to right, amplification of samples S7, 100% local cultivar 'Caaveiro'; S8, 95% commercial flour (type B) with 5% 'Caaveiro' flour; S9, 75% commercial flour (type B) with 25% 'Caaveiro' flour; S10, 75% commercial flour (type C) with 25% 'Caaveiro' flour; followed by their duplicates and a negative control, water, for each SSR (M1–M5). Purple lines show the manual threshold set for each assay.

the proportion of 'Caaveiro' and type A flours. The maximum peak heights of the specific alleles in 100% type A flour (192, 194, 208, 214, 222 and 230) are presented in Figure S5a, and those in 100% 'Caaveiro' (250 and 252) in Figure S5b. The height of the allele peak decreased as the proportion of that allele in the mixture was reduced (Figure S5c, d, e, f, g, h, i and j). Because the amount of 'Caaveiro' in the mixture increased as that of type A flour decreased, the peaks corresponding to alleles specific to 'Caaveiro' increased in height, while those specific to type A flour gradually decreased (on the left side of the figure are the alleles representing type A flour, and on the right are those representing 'Caaveiro'). The peak heights began to equalise when type A flour constituted 60% of the mixture (Figure S5k) and were fully balanced in the 50:50 mixture (Figure S5l). From this value, it is clearly seen how the peak heights of alleles specific to type A flour decreased, while those of alleles specific to 'Caaveiro' increased (Figure S5m, n, ñ, o). When the percentage of 'Caaveiro' rose to 75% (Figure S5p), all the alleles of type A flour were present, but their peaks were very low. In the 85% 'Caaveiro' mixture, the alleles of type A flour were more weakly amplified, but all of them were detectable (Figure S5q, r, s, t). The quantification of 'Caaveiro' in flour blends containing 100%, 75%, 50% and 25% 'Caaveiro' and 100% type A flour could also be performed in the same amplification (Fig. 4), specifically using alleles 250 and 252. The quantification of 'Caaveiro' in flour blends can be also determined in the same amplification (Fig. 4) when we represent flours with 100% 'Caaveiro', 75% 'Caaveiro', 50% 'Caaveiro', 25% 'Caaveiro' and 100% type A flour, specifically for alleles 250 and 252.

ddPCR

Droplet digital polymerase chain reaction amplifications of Xgwm0312 and Xgwm0099 showed highly significant R-squared values in the blends tested (Table 4, Figs 5 and 6). Xgwm0312 showed R^2 over 0.8 in both

Table 3 Number of alleles detected in different wheat varieties by using eighteen microsatellites, quality of the amplification, capacity to distinguish between seventeen varieties and ability to discriminate three types of commercial flours (A, B and C) and flour mixtures containing the autochthonous wheat 'Caaveiro'

Marker	No. of alleles detected in this study	Number of alleles (unique alleles) (Ruiz *et al.*, 2012)	Allelic rank	Alleles	Amplification quality in agarose	Amplification quality in the sequencer	Ability to distinguish cultivars	Ability to discriminate flours mixtures
Xgwm0148	8	12 (4)	138–164	89, 138, 140[2], 142, **144[1,2,3]**, 146, 160[1,3], 164	Good	Good	Yes	No
Xgwm0155	6	16 (3)	139–147	139, **141[1,2,3]**, 143[1,2,3], 145[1,2], 147, 149	Good	Good	Yes	No
Xgwm0156	11	20 (4)	283–317	283[1,2,3], 287[1,2,3], **289[2,3]**, **291**, 293, 296, 300, 312[1,2], 315[1], 317[1], 319	Good	Good	Yes	Yes+
Xgwm0186	8	28 (15)	117–137	100, 117, **119[1]**, 121[1,2,3], 123, 127[1,2,3], 129, 137[1,2,3]	Good	Good	Yes	No
Xgwm0234	12	19 (9)	198–245	198, **201[1,2,3]**, 224, **226[1]**, 228, 230, 234, 236, 238[1,2,3], 240[1,2,3], 244	Good	Good	Yes	No
Xgwm0312	27	26 (4)	184–250	184, 192[1], 194[1], 196, 208[1,2], 210, 212, 214[1,2], 218[2], 220, 222[1,2,3], 224, 226, 228, 230[1,2], 232, 235, 237, 239, 241, 243, 245, 248, **250, 252**, 256, 258	Good	Good	Yes	Yes++
Xgwm0332	22	20 (4)	190–245	**190[1,2,3]**, 193[1,2,3], **195[3]**, 200[1,2], 204, 208[1,2,3], 211[3], 213, 217, 219, 221, 223, 225, 227, **229**, 231, 233, 236, 241[1], 245, 250, 256	Good	Good	Yes	Yes+
Xgwm0099	21	11 (3)	105–155	105[1,2,3], 107, **109**, 111, 113, 115[1], 122, 124, **127**, 130, 132, **134**, 136, 138, 140, 142, 144, **147**, 149, 153, 155	Good	Good	Yes	Yes++
Xgwm0570	13	15 (3)	96–159	**96**, 106, 136[1,2,3], 138, 141, 143, 145[1], 148[1,3], 151[1,2,3], 153, 155, 157, **159**	Good	Good	Yes	Yes+
Xgwm0577	24	41 (15)	128–219	128[1,2], 130, 134[1], 136, 138, 140, 142, 147, 150, 152[1], **155**, 157[1,2], 159[1,2], 162[1], 176, 203, 205, 207, 209, 211, 213, 215, 217, 219	Good	Bad	Yes	No
Xgwm0060	10	19 (5)	116–138	116, 118[1,2,3], 122, 126, 128, 130, **132**, 134, 136, 138	Good	Good	Yes	Yes++
Xgwm0088	6	17 (4)	141–151	141, **143[1]**, 145[2,3], 147[1,2,3], 149, 151[1]	Good	Good	Yes	No
Xgwm0513	17	4 (0)	148–213	148[1,2,3], **150[1,2,3]**, **154[1,2,3]**, 160, 162, 174, 184, 188, 189, 194, 200, 203, 205, 207, 209, **211[1,2,3]**, 213	Good	Good	Yes	No
Xgwm0389	15	14 (3)	114–151	114, 116[1,2], 118, **120[3]**, 122, 124, 126, 128, 131, 133[3], 135, 137, 139[1], 143[1,2,3], 151	Good	Good	Yes	No
BARC155	9	11 (4)	180–206	180, 184[1,2,3], 186, 188, 194, 198, **201**, 203, 206	Good	Good	Yes	No
BARC80	6	17 (6)	101–133	101, 103, 106[1,2], **109[1,2,3]**, 114[1,3], 133	Good	Good	Yes	No
WMC468	12	5 (1)	129–157	129[3], 131, 133[1,2,3], 135[1], 140, 144, 146[1,2], 148, 151, **153**, 155, 157[1,2,3]	Good	Good	Yes	Yes+
Xgwm0002	11	4 (0)	116–277	116[1,2,3], **118**, 120[1,3], 127, 220[1,2,3], **222**, 224[1,3], 231, 255, **257[1,2,3]**, 277	Good	Good	Yes	No

In bold, alleles of 'Caaveiro'; in bold and underlined, specific alleles of 'Caaveiro'.
1, Alleles of flour A; 2, alleles of flour B; 3, alleles of flour C.

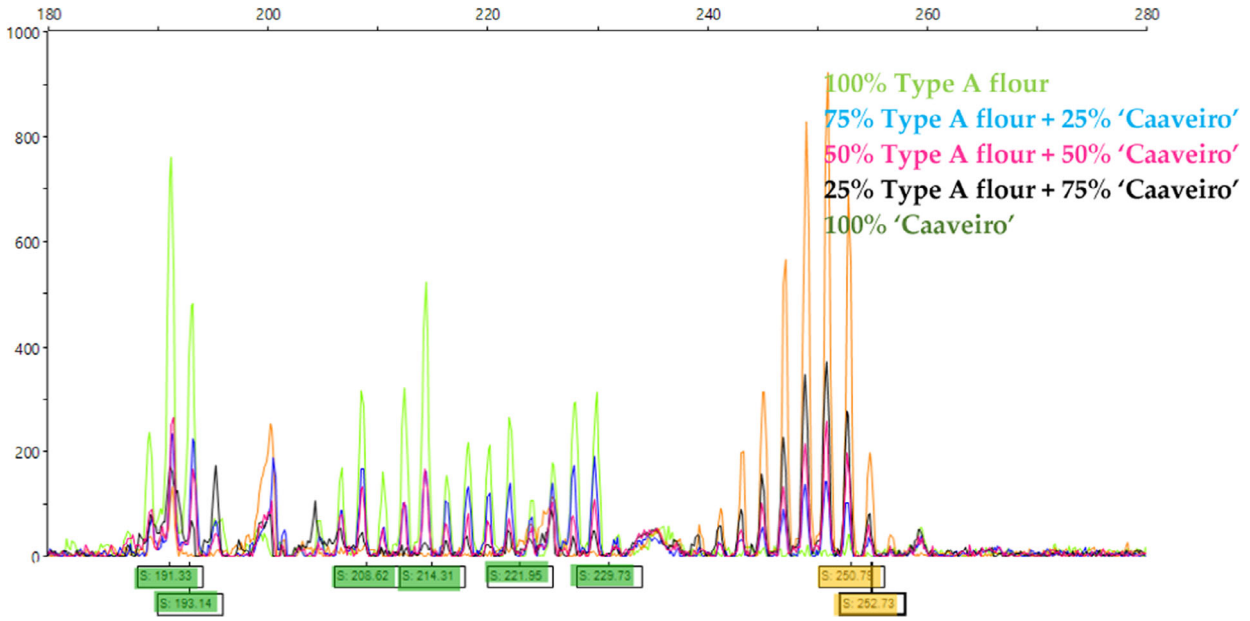

Figure 4 Comparison of the discriminant allelic profiles according to the proportion of 'Caaveiro' flour and type A flour by SSR Xgwm0312 (in green, alleles of type A flour; in orange, alleles of 'Caaveiro').

Table 4 *R*-squared values (R^2) of the linear regressions for the copies per microlitre and nanogram of samples of five SSRs in two trials with different dilutions and different mixes of 'Caaveiro' flour with commercial flours

Molecular marker	Trial	Dilution	R^2 for copies per µL sample	R^2 for copies per ng sample
Xgwm0312	1	D1	0.777	0.8907
Xgwm0312	1	D2	0.7401	0.9456
Xgwm0312	2	D3	0.6774	0.6672
Xgwm0312	2	D3	0.813[a]	0.8135[a]
Xgwm0099	1	D1	0.9882	0.9872
Xgwm0099	1	D2	0.9843	0.9639
Xgwm0099	2	D4	0.9515	0.9516
Xgwm0156	1	D1	0.8623	0.5934
Xgwm0156	1	D2	0.8935	0.1324
Xgwm0156	2	D3	0.4339	0.433
Xgwm0332	1	D1	0.8058	0.0031
Xgwm0332	1	D2	0.4781	0.4049
Xgwm0332	2	D4	0.6069	0.4781
Xgwm0570	1	D1	0.8395	0.0216
Xgwm0570	1	D2	0.9243	0.0025
Xgwm0570	2	D5	0.0049	0.0049

[a]Only for blends with flour type B.

trials (without flour type B in the second trial due to the lack of the blend with 5% of 'Caaveiro' flour). Xgwm0099 presented R^2 over 0.95 in both trials.

Amplification of Xgwm0156 and Xgwm0570 showed adequate R-squared values in the first trial for copies per microlitre of sample but not for copies per nanogram of sample. Xgwm0332 only showed adequate results for trial 1 and copies per microlitre sample with D1. Xgwm0156, Xgwm0332, and Xgwm0570 did not show adequate results in the second trial for blends with two commercial flours (B and C).

Xgwm0312 showed very reduced amplification in 'Caaveiro' wheat with respect to commercial cultivars, with a maximum of three copies per nanogram of sample for the two dilutions D1 and D2 in the first trial (Table 5) and for the D3 of the second trial (Table 6), meanwhile, in 25% 'Caaveiro' flour, there were thirteen copies per nanogram of sample (D3) to fourteen (D4) for commercial flour type A, four copies per nanogram of sample (D3) for type B and five copies per nanogram of sample (D3) for type C.

A maximum of 1291 to 6020 copies of Xgwm0099 per microlitre and 138 to 161 copies per nanogram of sample were amplified in each reaction (Table 6). Our results indicated that the number of copies per nanogram of sample in 25% 'Caaveiro' flour was seventy-three for samples D3 and D4 of commercial flour type A, eighty-nine D4 of type B and seventy-seven copies per ng sample D4 of type C.

Discussion

SSRs and genetic variability

In this study fourteen varieties of wheat were identified, the autochthonous wheat 'Caaveiro' (identical to

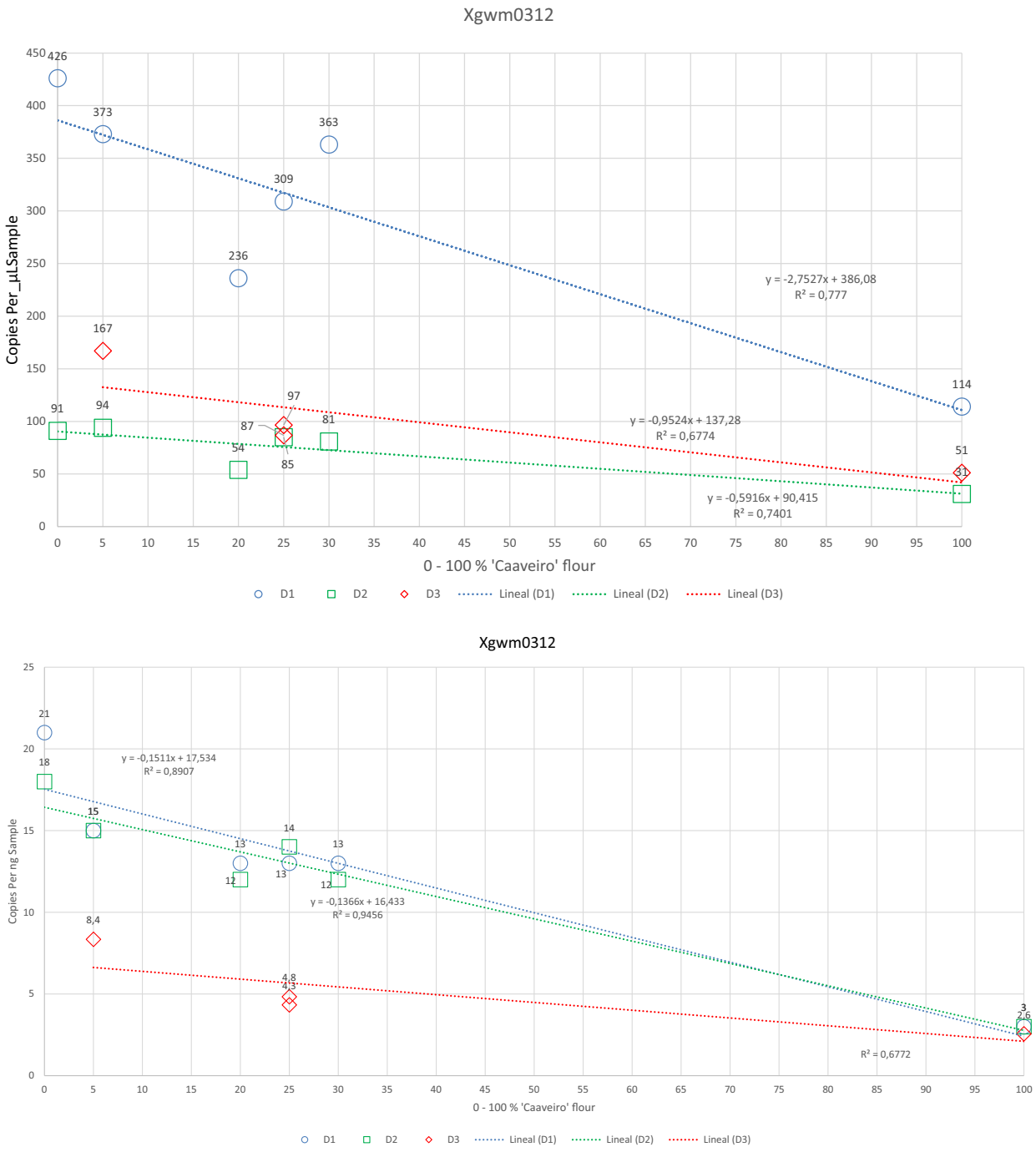

Figure 5 Linear regressions for Xgwm0312 by ddPCR for 'Caaveiro' flour and 3 commercial flours (type A, blue and green, types B and C, red) and some blends for the first trial (dilutions D1, blue; D2, green) and the second trial (dilution D3, red).

'Carral'), 'Callobre' and three commercial flours used for bread with eighteen markers that had previously been used in wheat studies in Spain (Ruiz *et al.*, 2012). Pasqualone *et al.* (1999) and Ruiz *et al.* (2012) both reported on the use of the microsatellite locus Xgwm0155; the latter obtained two alleles (135 and 136), while the present work described six alleles, ranging from 139 to 149 bp. Röder *et al.* (2002) also used

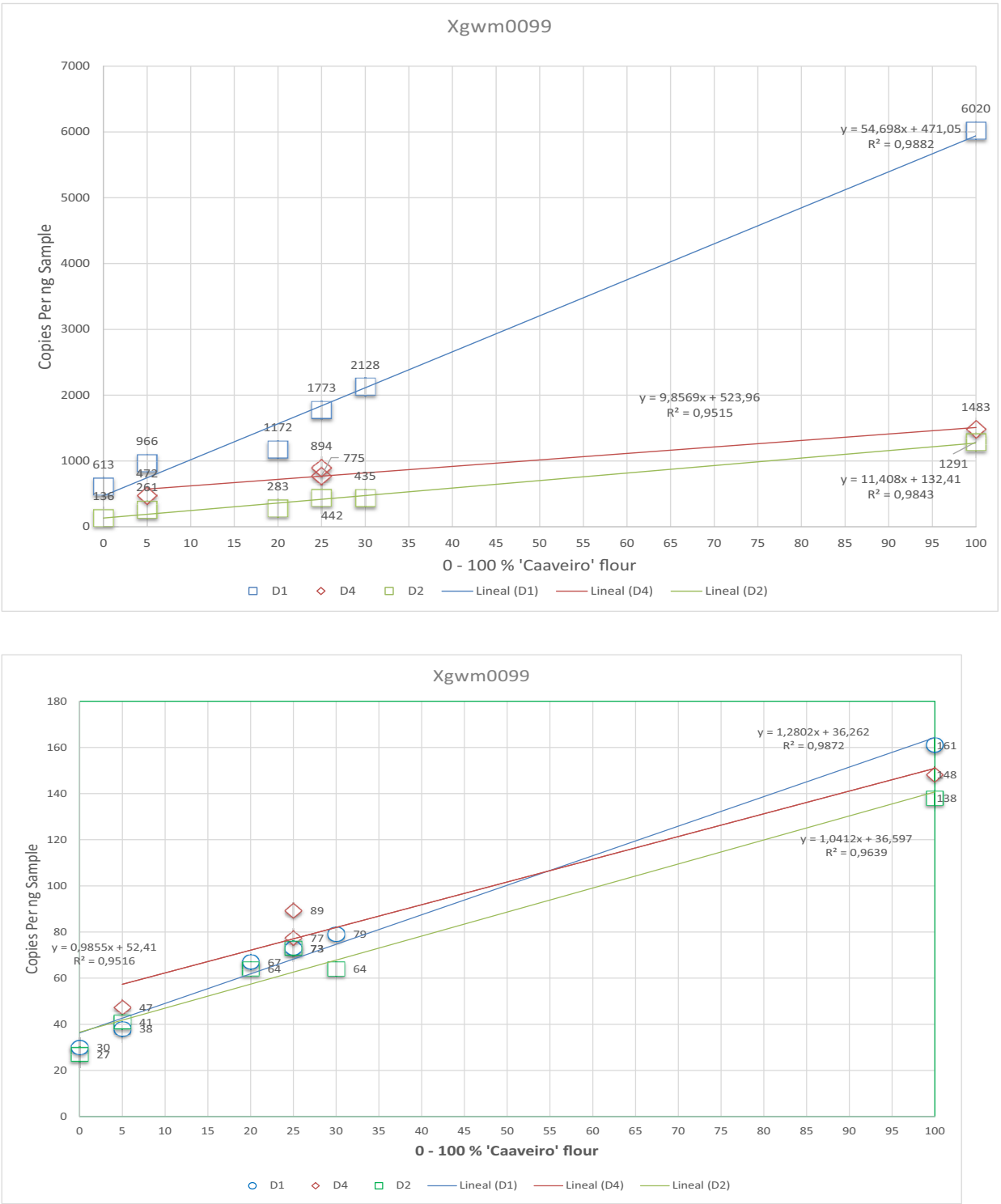

Figure 6 Linear regressions for Xgwm0099 by ddPCR for 'Caaveiro' flour and three commercial flours (types A, blue and green; B and C, red) and some blends for the first trial (dilutions D1, blue; D2, green) and the second trial (dilution D3, red).

Table 5 Amplification of Xgwm0312 by ddPCR in 'Caaveiro' flour and three commercial flours (types A, B and C) and some blends in the first trial (dilutions D1 and D2) and the second trial (dilution D3)

Trial	Dilution	Copies per μL sample	Copies per_ng Sample	Flours
1	D1	426	21	100% commercial flour (type A)
1	D1	373	15	5% 'Caaveiro' flour + 95% commercial flour (type A)
1	D1	236	13	20% 'Caaveiro' flour + 80% commercial flour (type A)
1	D1	309	13	25% 'Caaveiro' flour + 75% commercial flour (type A)
1	D1	363	13	30% 'Caaveiro' flour + 70% commercial flour (type A)
1	D1	114	3	100% 'Caaveiro' flour
1	D2	91	18	100% commercial flour (type A)
1	D2	94	15	5% 'Caaveiro' flour + 95% commercial flour (type A)
1	D2	54	12	20% 'Caaveiro' flour + 80% commercial flour (type A)
1	D2	85	14	25% 'Caaveiro' flour + 75% commercial flour (type A)
1	D2	81	12	30% 'Caaveiro' flour + 70% commercial flour (type A)
1	D2	31	3	100% 'Caaveiro' flour
2	D3	167	8	5% 'Caaveiro' flour + 95% commercial flour (type B)
2	D3	87	4	25% 'Caaveiro' flour + 75% commercial flour (type C)
2	D3	97	5	25% 'Caaveiro' flour + 75% commercial flour (type B)
2	D3	51	3	100% 'Caaveiro' flour (ecological management)

Table 6 Amplification of Xgwm0099 by ddPCR in 'Caaveiro' flour and three commercial flours (types A, B and C) and some blends in the first trial (dilutions D1 and D3) and the second trial (dilution D1)

Trial	Dilution	Copies per μL sample	Copies per_ng sample	Flours
1	D1	613	30	100% commercial flour (type A)
1	D1	966	38	5% 'Caaveiro' flour + 95% commercial flour (type A)
1	D1	1172	67	20% 'Caaveiro' flour + 80% commercial flour (type A)
1	D1	1773	73	25% 'Caaveiro' flour + 75% commercial flour (type A)
1	D1	2128	79	30% 'Caaveiro' flour + 70% commercial flour (type A)
1	D1	6020	161	100% 'Caaveiro' flour
1	D2	136	27	100% commercial flour (type A)
1	D2	261	41	5% 'Caaveiro' flour + 95% commercial flour (type A)
1	D2	283	64	20% 'Caaveiro' flour + 80% commercial flour (type A)
1	D2	442	73	25% 'Caaveiro' flour + 75% commercial flour (type A)
1	D2	435	64	30% 'Caaveiro' flour + 70% commercial flour (type A)
1	D2	1291	138	100% 'Caaveiro' flour
2	D3	47 241	47	5% 'Caaveiro' flour + 95% commercial flour (type B)
2	D3	77 488	77	25% 'Caaveiro' flour + 75% commercial flour (type C)
2	D3	8936	89	25% 'Caaveiro' flour + 75% commercial flour (type B)
2	D3	148 276	148	100% 'Caaveiro' flour (ecological management)

the same microsatellite and described nine alleles with a size ranging between 132 and 153 bp. Pasqualone *et al.* (1999) and Röder *et al.* (2002) both used the SSR Xgwm0577 and detected twenty-two alleles ranging from 126 to 214 pb. In our case, with this microsatellite, twenty-four alleles with a size between 128 and 219 bp were found. Ruiz *et al.* (2012) evaluated the SSRs Xgwm0332, Xgwm0156 and Xgwm0570. They detected nine, two and two alleles, respectively, for these loci, whereas we found twenty-two, eleven and thirteen, respectively.

Pasqualone *et al.* (2000) observed four alleles (130, 141, 154 and 164) for the microsatellite Xgwm0148, while in this work eight were detected (89, 138, 140, 142, 144, 146, 160 and 164).

The greatest allelic diversity found in the samples in general, and especially in type A flour, was related to its plurivarietal composition, which reflected the

mixture of wheats grown in that area. The markers chosen for wheat cultivar identification produced amplicon sizes below 320 bp in the present study and 300 bp according to other authors (Tilley, 2004; Fujita *et al*., 2009); moreover, Giancaspro *et al*. (2016) found that the size of wheat DNA fragments amplified from both durum and soft wheat was below 1000 bp.

ddPCR

We knew that 0% and 100% 'Caaveiro' flours did not contain any traces of other flours because sequencing of the same microsatellites amplified from these flours revealed unique alleles. Therefore, deviations from the mix could be due to the difficulty in obtaining the precise percentage of 'Caaveiro' in blended flours, in accordance with the results of microscopy (submitted for publication), where some blends showed deviations from the regression line due to incomplete mixing before the preparation of slides for counting specific starches present in 'Caaveiro'. As reported by Morcia *et al*. (2020), deviations could also be produced by sample processing or errors in the weighing of the samples and their correct homogenisation to quantify common wheat throughout the pasta production chain using cdPCR.

Moreover, it is known that the maximum number of amplicons from 100% 'Caaveiro' varied depending on the dilution required for each trial, which indicates that this reference must be used in each trial in order to determine the expected maximum number of amplicons. From this value, the number of amplicons can be calculated that would correspond to the minimum of 25% 'Caaveiro' needed to comply with the PGI 'Pan Galego', considering that the SSRs evaluated can also be amplified in commercial flours. In our study, two SSRs were able to determine the expected amplicons for the use of 'Caaveiro' in flour blends, independently of the dilution used, supported by the regression line obtained ($R^2 > 0.9$).

No previous references were found that discussed the use of SSRs in ddPCR for traceability and quantification of autochthonous cereal cultivars in flour blends, and a few were found based on SNPs and species-specific sequences (Perry & Lee, 2017; Morcia *et al*., 2020; Schulze *et al*., 2021).

As it was shown in a previous study on wheat blends (Perry & Lee, 2017), by using SNPs, not all selected markers performed well in ddPCR. In our evaluation of five SSRs, two of them were suitable for quantifying 'Caaveiro' wheat in flour blends.

Digital polymerase chain reaction (dPCR) was also successful in differentiating and quantifying wheat species in pasta by using primers to target the *Triticum* genus and common wheat in order to detect fraud in the flour used (Morcia *et al*., 2020). Durum wheat should be the only species used to produce pasta, and common wheat species must not exceed a maximum level of 3% to be considered contamination.

Quantitative estimates of wheat adulteration were found to depend on the DNA content and ploidy of cereal species and were also influenced by comminution, which is caused by overestimation of the proportion of less processed rye in more processed bread wheat and adulteration of durum wheat by common wheat by 1%–5%, resulting in underestimation of common wheat (Schulze *et al*., 2021), which we believe did not affect our results because, if this process was present, we knew the total amplicons for the pure flours for an accurate estimation.

Conclusions

This is the first report on the application of SSRs to the traceability of autochthonous wheat in flour blends by detecting specific alleles, which are amplified according to the proportion present, specifically, to ensure compliance with the minimum requirement of 25% Galician wheat of the PGI 'Pan Galego'. It is noteworthy that 7 (Wgwm0156, Wgwm0312, Wgwm0332, Wgwm0099, Wgwm0570, Xgwm0060 and WMC468) out of eighteen SSRs evaluated were useful in the identification of 'Caaveiro' in mixed flour, and three of them in particular had a higher differentiation capacity (Wgwm0312, Wgwm0099 and Xgwm0060), thanks to unique alleles not repeated in monovarietal flours. Moreover, the height of the allele peaks in the mixtures analysed showed a level of detection of 5%, which can be used as a fast-track method to determine the presence and the percentage of 'Caaveiro' in flour blends. The ddPCR technique has been shown to discriminate the level of 'Caaveiro' in a mixture of native wheat using two out of five microsatellites, which proves the usefulness of the technique for the traceability of 'Pan Galego', despite the high cost of this methodology.

Since dilutions give different maximum numbers of amplifications (nanograms per sample), samples need to be tested at the same dilution, using a minimum of 100% 'Caaveiro' as a reference, to know the maximum number of possible amplicons. Moreover, due to the variable composition of commercial wheat flours used for bread production, it is necessary to check both commercial and 'Caaveiro' wheat flours in pure form, in order to know the expected amplification level for the sample to be traced. The number of different commercial wheat flours used tends to be limited since industry supplies are mainly sourced from a few producers. Hence, the number of possible contributors to the blends reduces the flours to be tested in the case of a blind sample, which should be evaluated at about the minimum 25% required for the PGI 'Pan Galego'.

Therefore, in an unknown blend, firstly we would identify the presence of 'Caaveiro' by using the three most discriminant SSRs (Wgwm0312, Wgwm0099 and Xgwm0060) to detect their specific alleles; secondly, we would include in the same run 100% 'Caaveiro' and a blend containing 25% 'Caaveiro' to compare the height of the allele peaks (the unknown blend and the blend with 25% 'Caaveiro' should present the same height) and thirdly, we would proceed by ddPCR with Xgwm0312 and Xgwm0099 including the unknown and 5% and 100% 'Caaveiro' blends. Due to the high cost of ddPCR, the third step would be recommended when a confirmation of the minimum of 25% of 'Caaveiro' required in the 'Galician bread' is needed.

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Author contributions

Ana Ramos-Cabrer: Data curation (equal); methodology (equal); resources (equal); software (equal); writing – original draft (equal); writing – review and editing (equal). **María Nerea Fernández-Canto:** Data curation (equal); methodology (equal); resources (equal); software (equal); writing – original draft (equal); writing – review and editing (equal). **Fernando Almeida García:** Resources (equal); writing – original draft (equal); writing – review and editing (equal). **Ana Gorostidi:** Data curation (equal); methodology (equal); software (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal). **Matilde Lombardero-Fernández:** Funding acquisition (equal); project administration (equal); resources (equal); writing – original draft (equal); writing – review and editing (equal). **Mª Angeles Romero-Rodríguez:** Funding acquisition (equal); project administration (equal); resources (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal). **Santiago Pereira-Lorenzo:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

Ethics approval was not required for this research.

Peer review

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Data availability statement

Data available on request from the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Comparison of the quality of flour (a) and grain (b) amplification products, showing that there are no changes in the quality of the results.

Figure S2 Microsatellite (Xgwm0148) non-discriminant for the determination of flour mixtures by matching all the alleles of one monovarietal ('Caaveiro') with alleles of the other monovarietal (type A flour). The alleles in orange are of 'Caaveiro', the greens of Type A flours and in violet an allele that the two monovarietals present and, therefore, does not allow discrimination.

Figure S3 Microsatellite (Xgwm0312) with high flour discrimination power (++), since it does not present matching alleles in monovarietal flours. The orange alleles are from 'Caaveiro' and the green ones from type A flour.

Figure S4 Microsatellite (Wgwm0332) with less power of discrimination of flours (+), by presenting coincident alleles in monovarietal flours (in this case 190). The alleles in orange are of 'Caaveiro', the greens of Type A flour and in violet, those that cannot be discriminated.

Figure S5 Discriminant profiles of the flour mixtures 'Caaveiro' and type A flour by SSR Xgwm0312 (in green, alleles of type A flour; in orange, alleles of 'Caaveiro').

