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Genetic Diversity of Local Wheat (*Triticum aestivum* L.) and Traceability in the Production of Galician Bread (Protected Geographical Indication) by Microsatellites

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Abstract: Galician wheat (*Triticum aestivum* L.) serves as the foundational component of Galician bread, a traditional Spanish product granted the Protected Geographical Indication (PGI, EU quality scheme), which is primarily conserved at the Agricultural Research Center of Mabegondo (CIAM), Xunta de Galicia, A Coruña, Spain. This study evaluated 20 ecotypes and cultivars, in comparison to 4 Galician wheats and 14 commercial wheat varieties used as references. Seventeen simple sequence repeats (SSRs) were evaluated to elucidate their genetic structure, determine their origins, and differentiate them from commercial cultivars for traceability purposes. In total, 296 wheat plants were analyzed, revealing 156 unique genotypes, 13 of which were from commercial cultivars and 143 of which were from local cultivars and ecotypes. The SSR loci revealed 221 microsatellite alleles, with an average of 11 alleles per locus. Of these, 151 alleles were found in local cultivars and ecotypes, and 134 were present in commercial cultivars, with 65 and 50 alleles exclusive to each group, respectively. A Structure software analysis demonstrated substantial genetic differentiation (Fst = 0.26) between two primary clusters, RPP1 (comprising commercial cultivars, and two ecotypes, 41 and 43) and RPP2 (consisting of local cultivars, elite lines, and ecotypes). Moreover, neighbor-joining tree analysis and principal component analysis (PCA) confirmed the high differentiation between these clusters, highlighting the singularity of Galician wheat, which is useful for the traceability of Galician bread. Furthermore, the SSRs were effective in tracking the use of Galician wheat, which displayed specific Galician alleles, in flour, sourdough, and bread samples, corroborating previous findings even when a greater number of Galician ecotypes were included.

Keywords: simple sequence repeats (SSRs); *Triticum aestivum* L.; variability; local cultivars; ecotypes; germplasm bank; traceability; quality bread

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1. Introduction

Wheat (*Triticum aestivum* L., 2n = 6x = 42, 17 Gb, AABBDD genomes) is a monocotyledonous plant within the Poaceae family. It plays a pivotal role in human nutrition,

contributing to approximately 20% of the daily protein and caloric intake of the global population [1]. The unique viscoelastic properties of gluten proteins make wheat a fundamental ingredient in bread production [2]. Projections indicate that global wheat demand will increase by 50% by 2050 [3–5].

Bread wheat ranks as the second most extensively cultivated cereal globally. Spain, in particular, exhibits significant variability in its native bread wheat varieties, attributable to the country's diverse climates and soil types [6]. However, a considerable degree of admixture has been observed in certain bread wheat populations [1].

Galicia is a region in northwest Spain where local agricultural fields have persisted in producing bread wheat ecotypes related to the old tradition of making bread from native wheat [7]. Although it currently has little importance in wheat production at the national level, being responsible for 0.66% of the wheat area and cereal production [8], it has had high historical importance in the production of wheat and other cereals since the Neolithic period (2300–2070 BC), as demonstrated by pollen and carpological samples [9,10]. Lagasca and Clemente only referenced *Triticum turgidum* from Galicia in their herbarium at the beginning of the 19th century [11]; later, Planellas included other *Triticum* species in his work [12]. In the 20th century, three varieties of bare grain *Triticum* were described in a work on Galician Flora, which today would be related to *T. aestivum* L. ssp. *aestivum*, *Triticum turgidum* L. ssp. *Turgidum*, and *Triticum turgidum* L. *durum* (Desf.) [13], being the last citation referring to tetraploid wheat. However, none of these were coated grain; they were bare grains and tenacious rachises. Gadea, Sánchez-Monge, Sahuquillo and Fraga Vila and Urquijo [14–17] cited some other *Triticum aestivum* L. varieties from the Galician region, describing the different botanical varieties collected. From these last works [14–17], it can be summarized that the common names are related to some morphological or physiological quality of the variety, sometimes referring to the planting time or the area where they are grown. In the last century, no trace of the tetraploid wheat or hulled wheat varieties, including emmer and spelt wheat, mentioned in the previous works were reported [17].

One of the main concerns of the Mabegondo Agrarian Research Center (CIAM), Xunta de Galicia, A Coruña, Spain, has been to conserve and characterize Galician phylogenetic accessions. This has led CIAM to search for these ecotypes and collect samples of wheat bread from the Galician community, mainly from 2002 to 2014 [18]

These local bread wheat populations are housed in the CIAM germplasm bank, which currently contains 196 bread wheat ecotypes. Half of the accessions have been characterized using agronomic and grain quality descriptors [17]. Furthermore, all accessions have been described by their high molecular-weight glutenin subunits [17]. Over the last few years, different lines from these ecotypes have been selected at CIAM; five of these have been registered as local cultivars ('Callobre', 'Caaveiro', 'Castrexo', 'Miño' and 'Arzua', with registration numbers 20030328, 20130262, 20210292, 20210293 and 20210294, respectively). Galician wheat has a high protein and extensibility content, and a gluten content suitable for Galician bread, making it darker, with a more intense aroma and flavor [19]. Galician wheat produces weak or medium–strong flours, which is why they are usually mixed with stronger and more elastic flours.

Previous studies have demonstrated the effectiveness of various PCR techniques in traceability and contamination detection in wheat production, including, as follows: identifying and quantifying midge-tolerant wheat (MTW) seeds mixed with a midge-susceptible cultivar [20]; quantifying a 3% contamination level in the pasta production chain [21]; detecting common wheat in semolina and breads with detection thresholds of 3% and 5% [22]; genotype quantification in pasta production [23]; ensuring the exclusive use of durum wheat in pasta and traditional bread [24,25]; and identifying cultivars and detecting cross-contamination with a detection limit of 5% for specific contaminant varieties [26].

In a study utilizing SSRs, Su et al. [27] discovered that volunteer wheat, "a kind of wheat with weed characteristics, distributed widely in the main wheat-producing areas of China", exhibited greater variability compared to cultivated wheat, with the two displaying rather distant phylogenetic relationships. Further research has assessed the genetic diversity and population structure of wheat using SSRs [28–36] and inter-simple sequence repeats (ISSRs) [32,37]. Previous works at CIAM have provided greater knowledge of ecotypes and the registration of lines selected as local cultivars, which has allowed them to be used as part of the flour needed to make Galician bread under the Protected Geographical Indication (PGI) "Pan Gallego". 'Caaveiro' and 'Callobre' were previously identified with SSRs [38] in a combined strategy that included nutritional composition [39], microscopy [40] and neural networks [41] for the traceability of Galician wheat in Galician bread from the field to the table. However, there are 196 Galician ecotypes and local cultivars conserved at the CIAM that have not previously been evaluated with SSRs and could be used to produce Galician bread covered by the PGI Pan Gallego. A previous evaluation with 17 SSRs was successful in identifying 'Caaveiro' and 'Callobre' from commercial cultivars [38], even in mixed flours. Using Xgwm0312 in ddPCR, the percentage of 'Caaveiro' flour has been exactly quantified in blindly mixed flours. Quantification with ddPCR is of the same order as evaluation through microscopy [40] and neural networks [41], confirming the uniqueness of the Galician wheat 'Caaveiro'. Therefore, it is necessary to extend this previous work to a large number of Galician ecotypes, cultivars, and elite lines. The objective of this study was to assess the genetic diversity and structure of Galician wheat ecotypes and cultivars, determine their potential origin, differentiate them from commercial cultivars, and investigate their utility in traceability from field (ecotypes and cultivars) to fork (flour, sourdough, and bread) within the Galician Bread PGI production chain.

2. Materials and Methods

2.1. Plant Material

A total of 14 local wheat ecotypes, defined as farmer's populations by Villa et al. [42] (IDs 1, 5, 8, 10, 11, 13, 16, 17, 19, 22, 24, 30, 41, and 43), 4 elite lines (1910-4, 1967-5, E1L2, and E16L30) and 2 Galician cultivars ('Caaveiro' (prebase) and 'Callobre' (base), both registered as commercial cultivars (20130262 and 20030328)) were evaluated (Table S1). The previous inbred lines 1967-5, E1L2 and E16L30 have recently been registered as local cultivars (Arzua 20210294, Miño 20210293 and Castrexo 20210292, respectively). Winter wheat plants were grown in 2021 on the experimental farm (43°14′36″ N–8°16′24″ W) of CIAM-AGACAL (Agricultural Research Center of Mabegondo, Xunta de Galicia, A Coruña, Spain).

This study included the following 14 commercial cultivars previously evaluated by Ramos Cabrer et al. [38]: 'Enebro'; 'Nogal'; 'Rebelde'; 'Ovalo'; 'Valbuena'; 'Montecarlo'; 'Algoritmo'; 'Basilio'; 'Tocayo'; 'Radia'; 'Atomo'; 'Sensas'; 'Acorazado'; and 'Alhambra'. The study also included the following four Galician references: 'Caaveiro'; 'Carral'; 'Trigo Pais'; and 'Callobre'.

A total of 296 plant DNA samples, extracted from leaves, were tested following the methodology described by Ramos-Cabrer et al. [38]. This included 10 seedlings from each of the 14 ecotypes (resulting in 138 samples, with two not successfully amplified); 100 seedlings from the 4 elite lines (1910-4, 1967-5, E16L303, and E1L2); 20 samples from each of the 2 cultivars (yielding 40 samples); and 1 sample from each of the 14 commercial cultivars and 4 Galician references (totaling 18 samples) that did not show intracultivar variation [38].

2.2. Wheat Flour, Sourdough and Bread

To ensure traceability, an evaluation was conducted on 38 types of wheat flour, including 23 commercially available varieties, 6 of Galician origin, and mixed flours (refer to Table S1). Additionally, two sourdough samples were assessed, one commercially available sample, and the other of Galician origin. Furthermore, the evaluation included 18 bread samples, consisting of 10 commercial breads, 3 Galician breads, and 5 breads made with blended flours. Amplification for all SSRs could not be obtained for each bread sample.

2.3. DNA Isolation and PCR Conditions

DNA extraction. DNA extraction was performed using 0.5–0.75 g of young leaves in a E.Z.N.A.®Plant DNA Kit (OMEGA Bio-Tek Inc., Norcross, GA, USA) and a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The extracted genomic DNA was quantified using a NanodropTM ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and subsequently diluted to 20 ng/µL. The same kits were utilized to extract DNA from the flour and sourdough. However, for bread, the Speedtools Food DNA Extraction Kit (Biotools B&M Labs., S.A., Madrid, Spain) was used.

Microsatellites. For these analyses, 17 SSRs were selected that exhibited high polymorphism in previous studies conducted in Spain [38,43] (Table 1). The SSRs were amplified in 5 multiplexed PCRs using a fluorophore-labelled primer: FAM, NED, PET, or VIC (PE Applied Biosystems, Warrington, UK).

Table 1. Alleles in bp for 17 microsatellites (SSRs) for 156 unique genotypes of local Galician ecotypes/cultivars and commercial wheat cultivars, and 38 flour, 2 sourdough, and 18 bread samples.

SSR (Locus)	Allelic Rank [43]	No. Alleles in Previous Works in Spain [38]	Alleles in Previous Works in Spain [38]	Common Alleles (Present Study)	Exclusive Alleles (Present Study)	Exclusive and Previously Unreported Alleles in Flours, Sourdoughs, and Breads
Xgwm0148	138–164	8	89, 138, 140, 142, 144, 146, 160, 164	142, 144, 146	89 [2], **138** [1], 140 [1], 160 [1], **164** [1]	140 [1], 160 [1], 162 [3,4], 164 [1], 166 [1,3,4]
Xgwm0155	139–147	6	139, 141, 143, 145, 147, 149	**141**, 143, 145, 147	139 [2], 149 [1]	124 [1,3,4], 128 [1,3,4]
Xgwm0156	283–317	11	283, 287, 289, 291, 293, 296, 300, 312, 315, 317, 319	287, 289, 291, 312	283 [1], 293 [2], 296 [2], 300 [1], 319 [2]	283 [1], 300 [1], 315 [1,3], 317 [1,3], 321 [1,3,4]
Xgwm0186	117–137	8	100, 117, 119, 121, 123, 127, 129, 137	119, 121	**100** [1], 117 [2], 123 [2], 127 [1], **129** [1], **137** [1]	100 [1], 117 [2], 123 [2], 127 [1], 129 [1], 137 [1]
Xgwm0234	198–245	12	198, 201, 224, 226, 228, 230, 234, 236, 238, 240, 242, 244	201, **226**, 228, 234, 236, 238, 240, **242**, **244**	**198** [1], 224 [2], 230 [2]	198 [1]
Xgwm0312	184–250	27	184, 192, 194, 196, 208, 210, 212, 214, 218, 220, 222, 224, 226, 228, 230, 232, 235, 237, 239, 241, 243, 245, 248, 250, 252, 256, 258	184, **220**, 222, 230, **235**	192 [1], **194** [1], 196 [1], **208** [1], 210 [2], 212 [2], 218 [1], 224 [2], 226 [2], 228 [2], 230, 232 [2], 237 [2], 239 [2], 241 [2], 243 [2], 245 [2], 248 [2], 250 [2], 252 [2], 256 [2], 258 [2]	192 [1], 194 [1], 196 [1], 208 [1], 214 [3], 218 [1], 228 [2], 241 [2], 243 [2], 250 [2], 252 [2]

Table 1. *Cont.*

SSR (Locus)	Allelic Rank [43]	No. Alleles in Previous Works in Spain [38]	Alleles in Previous Works in Spain [38]	Common Alleles (Present Study)	Exclusive Alleles (Present Study)	Exclusive and Previously Unreported Alleles in Flours, Sourdoughs, and Breads
Xgwm0332 (A/B)	190–245	22	190, 193, 195, 200, 204, 208, 211, 213, 217, 219, 221, 223, 225, 227, 229, 231, 233, 236, 241, 245, 250, 256	A (190, 193, 195)/B (200, 211, 221, 236)	B (204 [2], 208 [1], 213 [2], 217 [2], 219 [2], 223 [2], 225 [2], 227 [2], 229 [2], 231 [2], 233 [2], 241 [1], 250 [1], 256 [2])	B (206 [3,4], 208 [1], 217 [2], 229 [2], 233 [2], 241 [1], 250 [1])
Xgwm0570	96–159	13	96, 106, 136, 138, 141, 143, 145, 148, 151, 153, 155, 157, 159	136, 143, 145, 148, 151, **153**	**106** [1], **138** [1], 141 [2], 155 [2], 157 [2], 159 [2]	106 [1], 136, 138 [1], 155 [2], 159 [2]
Xgwm0577	128–219	24	128, 130, 134, 136, 138, 140, 142, 147, 150, 152, 155, 157, 159, 162, 176, 203, 205, 207, 209, 211, 213, 215, 217, 219	140, **150**, 152, 155, **157**, 159, 162, 203, 205	**128** [1], **130** [1], 136 [2], 138 [2], 142, 176 [2], 207 [2], 209 [2], 211 [2], 213 [2], **215** [1], **219** [1]	128 [1], 130 [1], 134 [1,3], 136 [2], 142, 147 [1,3], 207 [2]
Xgwm0060	116–138	10	116, 118, 122, 126, 128, 130, 132, 134, 136, 138	128, 134, 136, **138**	118 [1], **122** [1], 126 [1], 130 [2], 132 [2]	116 [1,3], 118 [1], 120 [3,4], 126 [1], 132 [2]
Xgwm0088	141–151	6	141, 143, 145, 147, 149, 151	143, 145	141 [2], 147 [1], 149 [1], **151** [1]	147 [1], 149 [1], 151 [1]
Xgwm0513 (A/B)	148–213	17	148, 150, 154, 160, 162, 174, 184, 188, 189, 194, 200, 203, 205, 207, 209, 211, 213	A (148, 150, 154)/B (211, 213)	A (160 [1], **162** [1])/B (188 [1], **189** [1], 200 [2], 203 [1], 205 [1], 207 [2], 209 [2])	A (160 [1], 162 [1], 174 [3]/B (184 [1,3], 188 [1], 194 [1,3], 203 [1], 205 [1])
Xgwm0389	114–151	15	114, 116, 118, 120, 122, 124, 126, 128, 131, 133, 135, 137, 139, 143, 151	116, 118, 120, 122, 133, 135	114 [2]124 [2], 128 [2], 131 [2], 137 [1], 139 [1], 143 [1]	126 [1,3], 139 [1], 143 [1], 151 [1,3]
BARC155	180–206	9	180, 184, 186, 188, 194, 198, 201, 203, 206	184, 201, 203	**180** [1], **186** [1], **188** [1], 194 [2], 198 [2], 206 [2]	188 [1]
BARC80	101–133	6	101, 103, 106, 109, 114, 133	106, 109	101 [2], 103 [2], 114 [1]	114 [1], 118 [3,4]
WMC468	129–157	12	129, 131, 133, 135, 140, 144, 146, 148, 151, 153, 155, 157	133, **135**, 151, **155**, 157	**129** [1], 140 [2], 144 [2], 146 [1], 148 [1], 153 [2]	129 [1], 131 [3], 146 [1], 153 [2]

Table 1. *Cont.*

SSR (Locus)	Allelic Rank [43]	No. Alleles in Previous Works in Spain [38]	Alleles in Previous Works in Spain [38]	Common Alleles (Present Study)	Exclusive Alleles (Present Study)	Exclusive and Previously Unreported Alleles in Flours, Sourdoughs, and Breads
Xgwm0002 (A/B)	116–277	11	116, 118, 120, 127, 220, 222, 224, 231, 255, 257, 277	A (116, 118, 120, 127)/B (220, 222, **224**, 257)	B (231 [1], 255 [1])	B (231 [1], 277 [3])

[1] Exclusive alleles for commercial cvs.; [2] Exclusive alleles for local cvs. and ecotypes; [3] Exclusive alleles in flours, sourdoughs, and breads; [4] alleles not reported previously. In bold, rare alleles in commercial cvs.; underlined, rare alleles in local cvs. and ecotypes.

PCR conditions and allele detection. The amplification conditions were as follows: 5 min at 94 °C followed by 35 cycles of 30 s at 95 °C for 30 s, 90 s at the annealing temperature specific to the multiplex set and 1 min at 72 °C, and final extension for 30 min at 60 °C.

The amplified products were then diluted with water. A volume of 2 µL of the diluted amplification product was added to 0.12 µL of the 600LIZ size standard (Applied Biosystems, Foster City, CA, USA) and 9.88 µL of formamide. The sizes of the alleles were detected using Peak Scanner™ v 1.0 software (Applied Biosystems).

2.4. Data Analysis

Ecotypes and cultivars. The sample size, number of alleles, number of effective alleles, information index, observed heterozygosity, expected and unbiased expected heterozygosity, and fixation index were estimated using GenAlEx 6.2 [44].

Considering unique genotypes, Bayesian analysis was conducted utilizing Structure software. version 2.3.4. [45,46]. This analysis employed the admixture model with unlinked loci and correlated allele frequencies, as outlined in the studies by Ruiz et al. [43], Pereira-Lorenzo et al. [47], and Porras-Hurtado et al. [48]. The last study recommended a minimum of 20 iterations, although 30 iterations were used in this particular study to estimate the ancestry membership proportions of a population. Structure software uses Markov chain Monte Carlo (MCMC) estimation by randomly assigning individuals to a pre-determined number of groups. Variant frequencies are then estimated for each group, and individuals re-assigned based on these estimates. This process is repeated many times, often up to 200,000 iterations during the burning process, followed by 1,000,000 MCMC (Monte Carlo Markov chain) replicates [48–50], leading to reliable allele frequency estimates and membership probabilities for each population [48]. We computed K values ranging from 1 to 15 [46,47] representing unknown reconstructed panmictic populations (RPPs) of genotypes. The options popinfo = 0 and popflag = 0 were selected, assuming that the sampled genotypes were of an unidentified origin [47]. These genotypes were probabilistically assigned to RPPs based on a probability of membership (qI) of 80% [47,49,50]. Genotypes with a lower probability were considered admixed. The second-order change of the likelihood function, divided by the standard deviation of the likelihood (ΔK), was also calculated to determine the best K value supported by the data [51]. This calculation was performed using Structure Harvester 8.

GenAlEx 6.2 [44] was used to perform analysis of molecular variance (AMOVA) [52,53] considering the RPPs obtained by the Structure software.

For the unique genotypes of ecotypes and cultivars, DARwin software, version 6.0.010 was employed to construct a dissimilarity tree [54–56] using the option "Dissimilarity for Allelic data", followed by unweighted neighbor-joining to build a tree with all genotypes.

Factorial component analysis (FCA) was performed using the principal components (PC) method in SPSS Version 28 [57]. For each unique genotype of the ecotypes (up to 20 samples) and commercial cultivars (up to 10 samples), the frequency of each allele was assigned to a variable, with values of 1 and 0 indicating the presence and absence of the allele, respectively. PCs were estimated based on the variance–covariance matrix of the allele frequencies [58–60] using SPSS software (version 28) [57].

Graphical representations of the PCs included the genetic structure and type of material evaluated with SSRs.

Flour, sourdough, and bread. In order to evaluate the use of the 17 SSRs in traceability from the land (ecotypes and cultivars) to the table (flour, sourdough and bread) in the production of Galician bread (PGI), a second FCA was performed using the PC method in SPSS V.28 [57], including the unique genotypes obtained for 38 flour, 2 sourdough and 18 bread samples in the previous FCA. The frequency of each allele was again assigned to a variable, with values of 1 and 0 indicating the presence and absence of the allele, respectively.

3. Results

3.1. Genetic Diversity

In this study, 156 unique genotypes were identified (Table S1) from a total of 296 plant DNA samples. Among these, 13 were commercial cultivars ('Alambra' and 'Sensas' showed the same genotype), while the remaining 143 were local cultivars ('Caaveiro' and 'Carral' had the same genotype) and ecotypes.

'Caaveiro' and 'Caaveiro' (prebase) (seed used for certification purposes), 'Callobre' and 'Callobre' (prebase), E16L30 (cv. 'Castrexo'), and 1910-4 each presented a unique genotype. However, elite lines E1L2 (cv. 'Miño') and 1967-5 (cv. 'Arzua') exhibited variations for 7 and 1 SSRs, respectively. One sample misclassified as 'Caaveiro' (prebase) turned out to be 'Callobre'; however, it displayed alleles of 'Caaveiro' for 2 of the 17 SSRs.

All ecotypes showed more than one genetic profile, with populations 8, 11, and 22 having the lowest number of genotypes (8) and 1, 5, 10, 16, 17, 19, 24, and 30 having the highest (10); therefore, the clonality varied from 0 to 20%, with an average of 6.4%.

In this study, 221 alleles were identified. Of these, 151 were found in local cultivars and ecotypes, and 134 were present in commercial cultivars. Specifically, 65 alleles were exclusive to local cultivars and ecotypes, and 50 were unique to commercial cultivars. Additionally, 69 were classified as rare alleles in local cultivars and ecotypes, and 37 were rare in commercial cultivars (Tables 1 and S2).

The average number of alleles per locus was 11. The Xgwm0312 locus exhibited the highest level of polymorphism with 27 alleles, while BARC80 had the lowest, with 6 alleles.

The flour, sourdough and bread samples collectively presented 149 alleles (Tables 1 and S2). Of these alleles, eight had not been reported in previous studies (four in commercial flour, two in Galician bread, and two in both). Additionally, 14 exclusive alleles for local ecotypes and cultivars were present in the flour, sourdough, and bread samples.

The average observed and expected heterozygosities were 0.23 and 0.68, respectively. A minimum observed heterozygosity (Ho) of 0 was found for Xgwm0332-B and Xgwm0513-B, and a maximum of 1 was observed for Xgwm0234. The minimum expected heterozygosity (He) of 0.38 was noted for Xgwm0148, and the maximum He of 0.91 was recorded for Xgwm0312 (Table S2).

3.2. Genetic Structure, Dissimilarity of Allelic Data and Factorial Component Analysis

Genetic structure. A Bayesian analysis was conducted utilizing Structure software [45]. This analysis employed 17 SSRs to ascertain the genetic structure among 156 unique

genotypes. According to the DK criterion, the most likely estimation was K = 2 (Figure S1 and Table S1). This estimation was derived using Structure Harvester [61] and applied to a group of 155 genotypes from the total of 156, each with a *qI* > 80% (representing 99% of all genotypes). This analysis resulted in a strong differentiation into two primary groups of genotypes, also known as RPPs. The first group, RPP1, comprised 31 genotypes, which accounted for 19.87% of the total number of genotypes. This group included commercial cultivars and two populations (41 and 43) from Folgoso do Caurel, Lugo. The second group, RPP2, consisted of 125 genotypes, representing 80.13% of the total number of genotypes. An additional genotype with a *qI* > 70% was also considered to be part of this group. RPP2 exclusively included local cultivars, elite lines, and ecotypes (Figure 1 and Table S1).

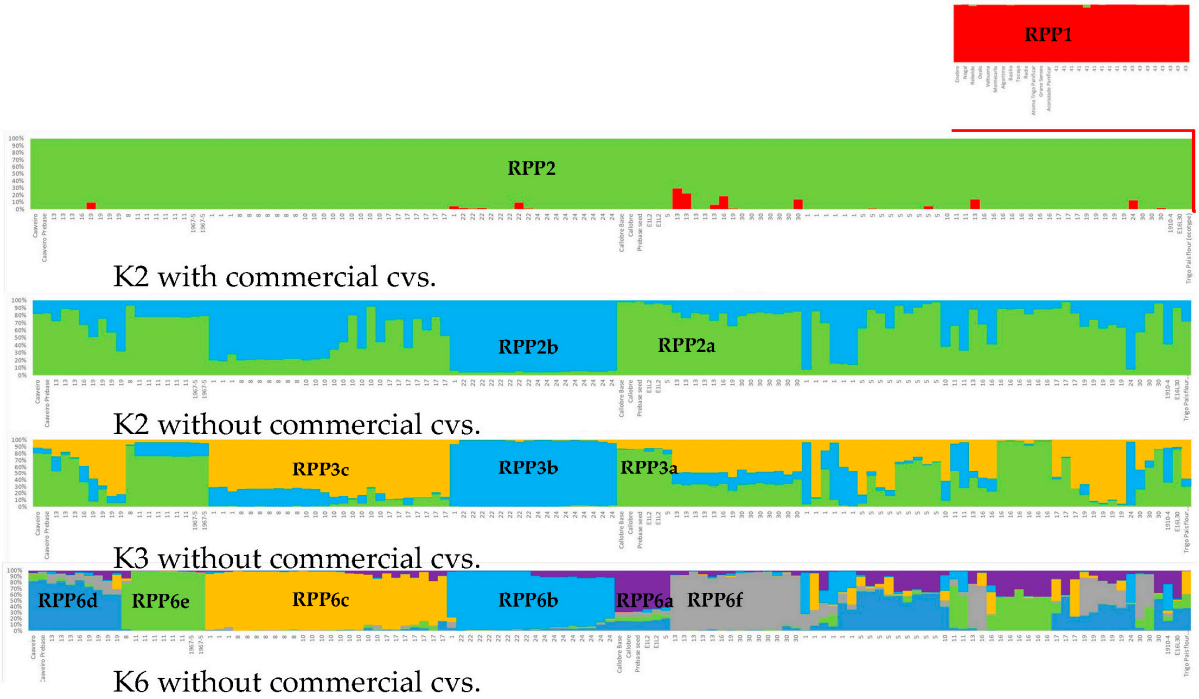

Figure 1. Bayesian analysis was conducted using 17 microsatellites (SSRs) with Structure software on 156 unique genotypes, including local Galician ecotypes/cultivars and commercial wheat cultivars, as well as 38 flour, 2 sourdough, and 18 bread samples. The genetic structure was analyzed for K = 2 with commercial cultivars (RPP1 in red), and for K = 2, 3, and 6 for local Galician cultivars and ecotypes, excluding commercial cultivars from the analysis.

The genetic differentiation between RPP1 (which included commercial cultivars and 2 ecotypes, 41 and 43) and RPP2 (comprising local cultivars, elite lines, and ecotypes) was significantly high, with an Fst value of 0.260 ($p < 0.01$).

The differentiation between commercial and local cultivars was attributed to the specific alleles present in each group (50 and 65, respectively). A higher number of these specific alleles in local cultivars were found in SSRs Xgwm0312, Xgwm0332 and Xgwm0577 (Table 1). The total number of alleles was also higher in local cultivars, with 151 compared to 134 in commercial cultivars. This trend was also observed in the number of effective alleles (Table S2).

The He was considerably higher (0.629 and 0.599 for RPP1 and RPP2, respectively) than the Ho (0.215 and 0.233 for RPP1 and RPP2, respectively).

A second Bayesian analysis was conducted on RPP2, which consisted of 125 local genotypes. This analysis excluded commercial cultivars and related Galician populations from RPP1. The analysis revealed the most probable subdivisions to be K = 2, 3, and 6 subgroups (Figure 1). For K = 2, the cultivars 'Caaveiro', 'Callobre', 'E1L2' and 1967-5 and

most of the Galician populations were grouped into RPP2a. This grouping differentiated populations 22 and 24 into RPP2b. K = 3 clustered most of the accessions of populations 8, 10 and 17 into RPP3c. K = 6 differentiated 'Caaveiro' (grouped into RPP6d) from 'Callobre' (grouped into RPP6a). Additionally, two more groups were formed: one comprising Galician populations 11 and 1967-5 (grouped into RPP6e), and another with populations 13 and 30 (grouped into RPP6f).

Dissimilarity of allelic data. The neighbor-joining (NJ) tree, which was based on the genetic distance matrix of 156 wheat genotypes, also grouped these genotypes in alignment with the structure for K = 2 (Figure 2). This grouping included commercial cultivars and ecotypes 41 and 43 from Lugo in a distinct branch, but these were not mixed. Elite line 'E1L2' was genetically closer to cultivar 'Callobre'. This can be attributed to their common origin from the original ecotype 'Callobre', from which both were selected in A Coruña. Conversely, the elite line 'E16L30' and 'Caaveiro', which also originated from the 'Callobre' ecotype, were more distant. This was also the case for the elite line from Lugo, '1910-4'.

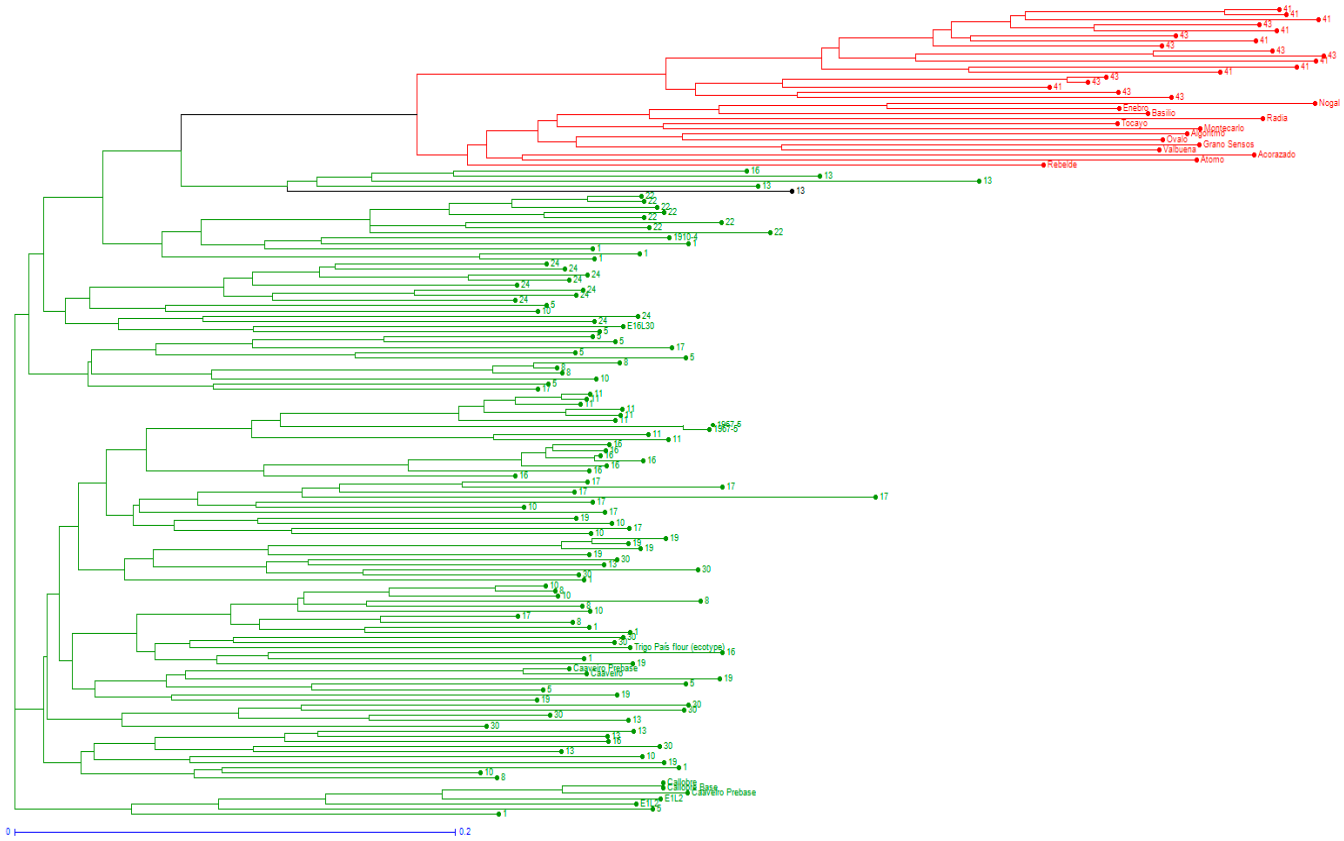

Figure 2. Neighbor-joining tree constructed using 17 SSRs across 156 unique wheat genotypes. The commercial cultivars and Galician ecotypes associated with RPP1 (K = 2), identified with Structure software, are highlighted in red. In green, local ecotypes and cultivars.

FCA. When a factorial correspondence analysis (FCA) was performed using the PC method, PC1 and PC2 accounted for 21% of the total variation, confirming significant differentiation between local cultivars and Galician populations from the commercial cultivars in the first PC. The substructure (K3 in RPP2) was primarily observed in the second PC (Figure 3). A total of 63 exclusive alleles discriminated local ecotypes and cultivars in negative PC1, among which two had a stronger influence (Tables 1 and S1), as follows: 153 for WMC468; and 132 for Xgwm0060. Fifty exclusive alleles for commercial cultivars had a positive influence in PC1, including, as follows: 160 for Xgwm0513A; 283 for

Xgwm0156; 203 for Xgwm0513B; 148 for WMC468; 250 for Xgwm0332B; 192 for Xgwm0312; 147 for Xgwm0088; and 196 for Xgwm0312.

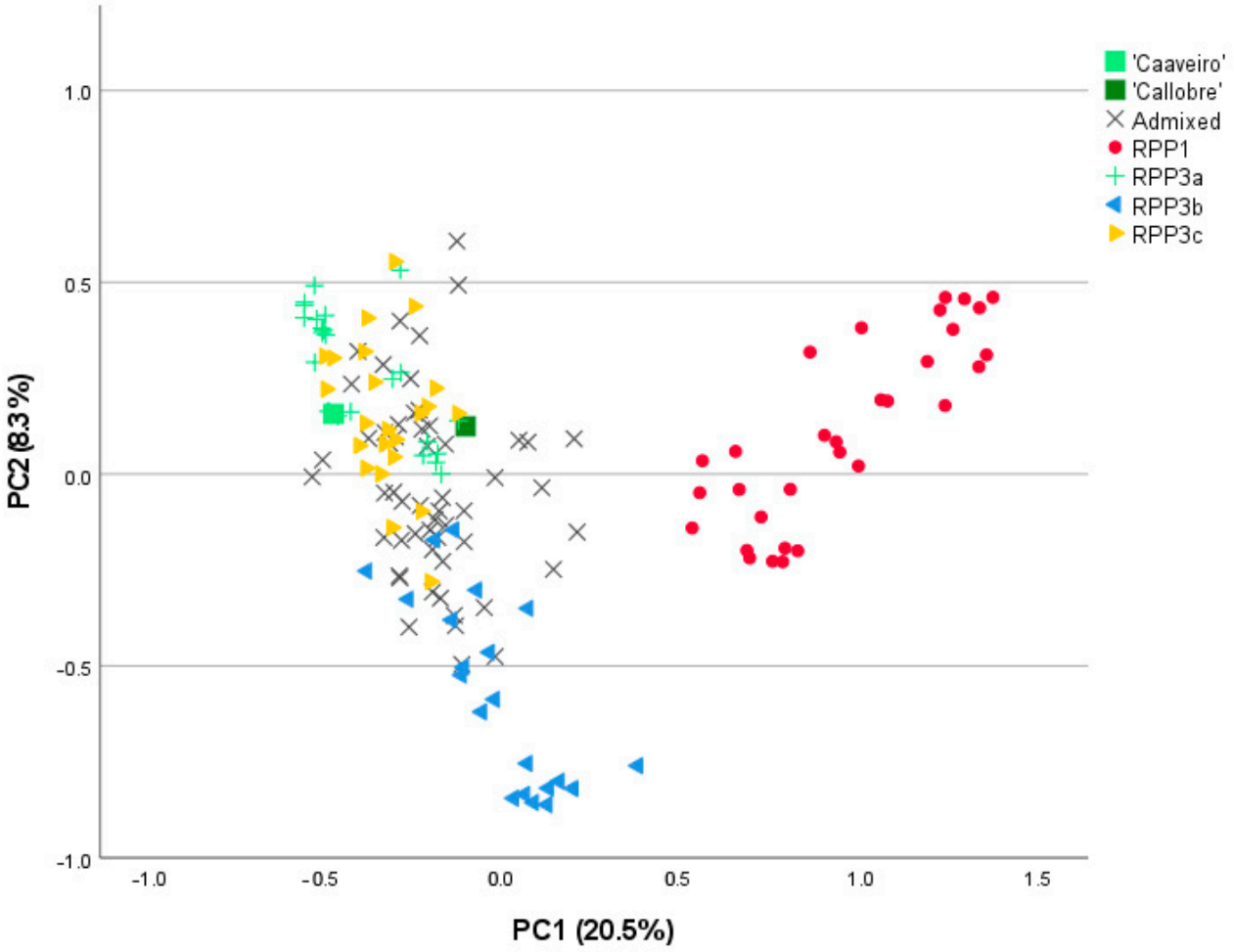

Figure 3. Factorial component analysis (FCA) was conducted using the principal components method in SPSS V.22 to examine the allele variation across 17 SSRs for a set of 156 genotypes. Genotypes were commercial cultivars and Galician ecotypes 41 and 43, which were associated with RPP1 for K = 2 and were obtained by Structure software in conjunction with the commercial cultivars. Additionally, three subgroups for K = 3 of local cultivars and Galician ecotypes (RPP3 a, b, and c) were also included in the analysis.

This study identified multiple genotypes for each ecotype (Table S1), which were related to their geographical origin at the provincial level (only shown for PC1, Figure 4). This genetic variation was particularly evident for populations 41 and 43, classified in RPP1 for K = 2 when commercial cultivars were included in the analysis (Figures 1 and 2), and which were collected in Lugo. This province exhibited the highest genetic variability in the study, as it was also the origin of the ecotypes classified in RPP3a, b, and c. In contrast, the provinces of Pontevedra and Ourense demonstrated the lowest genetic variations, with the former being the origin of one ecotype clustered in RPP3a, and the latter grouping one genotype in RPP3c. This was also associated with a lower number of entries from these provinces in this study. Moreover, PC1 (Figure 4) also shows how these ecotypes are genotypically diverse. For example, the genotypes from ecotype 11 classified in RPP3a were separate from those considered as admixed. Additionally, genotypes from ecotypes 41 and 43 (clustered in RPP1) were intermixed in the PC1, indicating the difficulty in differentiating them. We represented Galician local cultivars and elite lines with the commercial cultivars to show not only the significant genetic differentiation of 'Caaveiro'

and 'Caaveiro' (prebase) (seed used for certification purposes), 'Callobre' and 'Callobre' (prebase), E16L30 (cv. 'Castrexo'), and 1910-4, but also the variation found for the elite lines E1L2 (cv. 'Miño') and 1967-5 (cv. 'Arzua'). The same were misclassified as 'Caaveiro' (prebase) placed between 'Caaveiro' and 'Callobre' for PC1 because these showed the genetic profile of 'Callobre' with alleles of 'Caaveiro'.

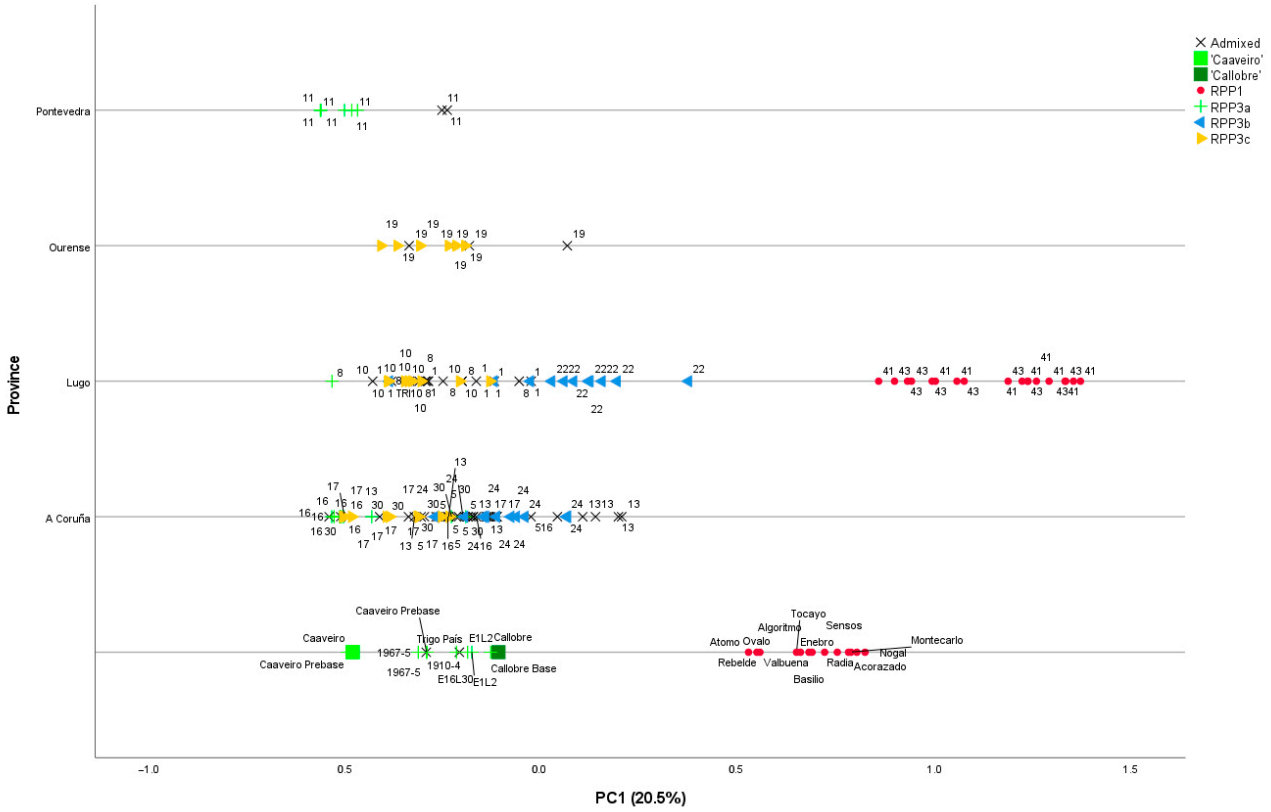

Figure 4. Factorial component analysis (FCA) was conducted employing the principal components method within SPSS V.22 software to examine the allele variation across 17 SSRs for a collection of 156 genotypes. Distribution of the genotypes categorized by province (PROV) attending to the PC1 and the RPP1 (comprising commercial cvs. and related Galician ecotypes) for K = 2 were obtained using Structure software. Additionally, the three subgroups of Galician local cultivars and ecotypes were identified for K = 3 are denoted as RPP3a, RPP3b, and RPP3c.

Most accessions of each ecotype were assigned to a specific RPP; however, some accessions were classified as admixed. For instance, populations 11 and 19 were from Pontevedra and Ourense, respectively (Figure 4 and Table S1). No samples from any other Galician population, apart from 41 and 43, were classified with the commercial cultivars (RPP1 for K = 2). However, when commercial cultivars were excluded, some accessions were classified into different RPPs than the majority. For example, for K = 2, Galician population 1 showed seven genotypes in RPP2b, one in RPP2a and one was admixed. Similarly, population 8 presented seven genotypes in RPP2b and one in RPP2a (Table S1).

3.3. Traceability of Galician Wheat in Flour, Sourdough and Bread Samples

The SSRs evaluated in this study were able to identify Galician ecotypes and cultivars, as well as their use in the production of flours, sourdoughs, and breads mandated by the PGI "Galician bread" [19]. A total of 14 exclusive alleles of local ecotypes and cultivars were present in flour, sourdough, and bread samples (Table 1 and Table S1), allowing for the traceability of the use of local wheats. Seventeen SSRs exhibited exclusive alleles for commercial cultivars, while sixteen SSRs (all except Xgwm0002) displayed exclusive

alleles for both local cultivars and ecotypes. The SSR Xgwm0513 had the highest number of exclusive alleles for commercial cultivars, with eight exclusive alleles, whereas Xgwm0234 and BARC80 each had only one exclusive allele. Regarding local cultivars and ecotypes, SSR Xgwm0312 presented the highest number of exclusive alleles, with 16 while Xgwm0088 had the fewest, with only 1 exclusive allele.

When FCA using the PC method was performed including ecotypes, commercial and local cultivars, and flour, sourdough, and bread samples, the three first PCs accounted for 33% of the variance (Figure 5). The local ecotypes from CIAM were distinctly differentiated on the negative side of PC1 from the commercial cultivars, which was attributable to the specific alleles identified in this study (Tables 1, S1 and S3), specifically the exclusive alleles for ecotypes and cultivars, mainly 153 for WMC468 and 101 for BARC80, among others (55) with a negative influence on the PC2. However, ecotypes 41 and 43 were exceptions, as they grouped closer to the commercial cultivars on the negative side of PC2, thereby corroborating the results presented in the neighbor-joining tree (Figure 2). Fifty exclusive alleles for commercial cultivars and 14 more found in commercial flours, sourdoughs, and breads had an influence on positive PC1, particularly 118 for Xgwm0060, 146 for WMC468, 127 for Xgwm0186, and 208 for Xgwm0332B.

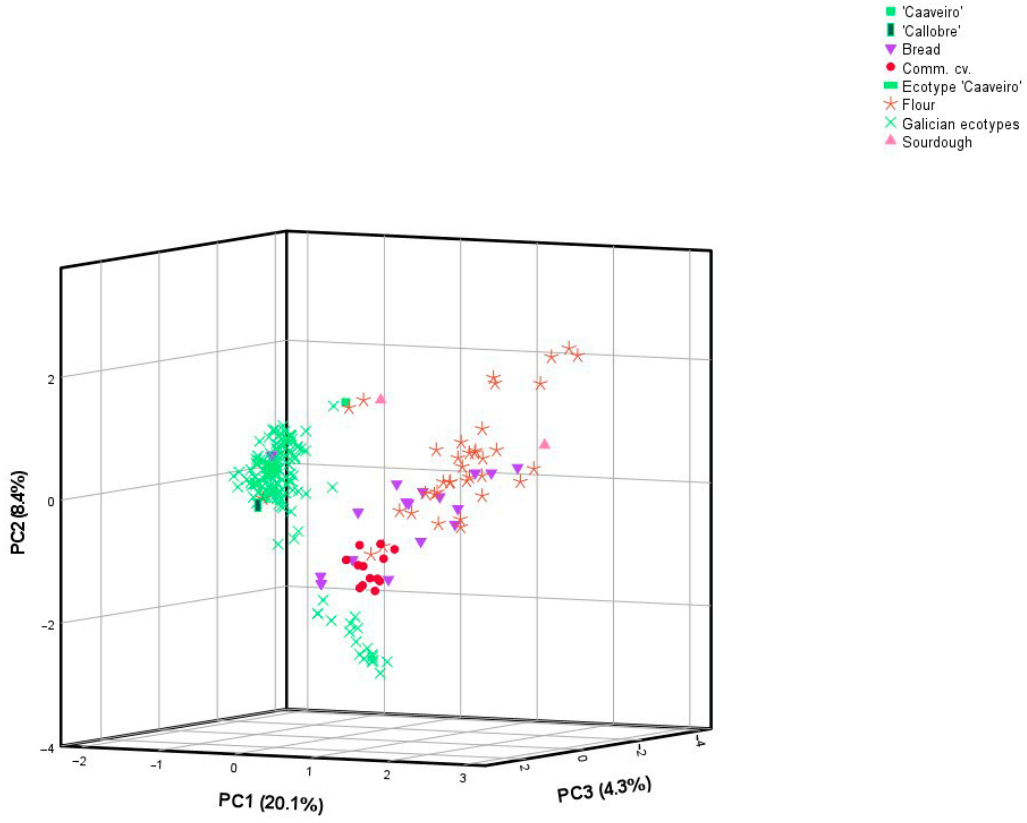

Figure 5. Factorial component analysis (FCA) was conducted using the principal components method within the SPSS V.22 software to examine the allele variation across 17 SSRs for a collection of 156 genotypes (commercial and local cvs. and ecotypes), 38 flours, 2 sourdoughs, and 18 breads.

Flour and bread produced with commercial cultivars were grouped together. Flour and bread made with a blend of Galician wheat were clustered between the Galician group and the commercial cultivars on the positive sides of axes PC1 and PC2 (Figure 5). The only exceptions to this were flour, sourdough, and bread made solely with Galician wheat, which were clustered with the Galician group (negative PC1 and positive PC2).

The 23 commercial flours analyzed showed exclusive alleles, as expected (Tables 1 and S1). The three Galician flours analyzed showed exclusive alleles from lo-

cal ecotypes and cultivars; however, the three purchased Galician flours only presented exclusive alleles of commercial wheats (Table S1). The commercial sourdough presented exclusive alleles from commercial cultivars, as expected, but the one made with 'Caaveiro' flour had Galician and commercial-exclusive alleles.

After analyzing 11 bakery breads, only 7 presented exclusive alleles from commercial cultivars, only 1 had exclusive alleles from local ecotypes and cultivars, and 3 showed alleles from both commercial and local ecotypes and cultivars (Tables 1 and S1). The bakery bread "Doval" only amplified for Xgwm0186, amplifying two exclusive alleles from commercial cultivars and the allele 119, which is present in 'Caaveiro' wheat, indicating that a blended flour was used at the bakery. One of three breads made with Galician flour presented exclusive commercial alleles, suggesting contamination during the bakery process. Finally, the flour breads prepared with blended flours presented exclusive alleles from both local and commercial ecotypes and cultivars.

4. Discussion

4.1. Genetic Diversity

The microsatellites chosen for this investigation exhibited polymorphism and demonstrated a pronounced genetic divergence between the commercial cultivars and the Galician populations and cultivars. This divergence has previously facilitated the tracing of Galician flour use in blends of Galician bread [38]. Furthermore, it validated the proficiency of SSRs in differentiating Galician wheats when the study incorporated a larger quantity of ecotypes and cultivars. Consequently, this facilitated the tracing of these wheats in mixed blends with commercial cultivars.

The observation of a lower Ho relative to the He across all loci aligns with the inbreeding traits of durum wheat originating from Ethiopia [29]. Prior studies have reported greater genetic diversity in durum wheat compared to bread wheat in landraces from various countries, including Oman [62] and Spain [1].

4.2. Genetic Structure, Dissimilarity of Allelic Data and Factorial Component Analysis

The genetic differentiation between Galician populations (RPP2) and commercial cultivars (RRP1), as indicated by an Fst value exceeding 0.26, implies a significant differentiation between the two groups. This level of differentiation is quite comparable to that observed in a study involving single-nucleotide polymorphisms (SNPs) from INIA–Uruguay bread wheat cultivars [63]. Moreover, it surpasses the values of genetic differentiation obtained through AMOVA among durum wheat populations from Ethiopia (12%) [29]. This differentiation is also considerably higher than that reported in various studies, including those by, as follows: Mondini et al. [64] in durum wheat (18.76%); Alipour et al. [65] in Iranian hexaploid wheat (15.56%); Amallah et al. [66] in durum wheat of the Mediterranean region (16%); Arora et al. [67] in Indian wheat cultivars (21.29%); and Ruiz et al. [43] in Spanish tetraploid wheat (20%).

There appears to be a distinct genetic pool that is representative of most Galician bread wheat ecotypes, in contrast to most of the other accessions from the Iberian Peninsula. This corroborates the results of Pascual et al. [1] using SNP and diversity array technology (DArT) markers, which identified a genetically distant group of western Iberian wheats. This observation aligns with the findings of Urquijo Zamora [17] in earlier studies that were predicated on the high molecular-weight glutenin subunit profile of the endosperm. Pascual et al. [1] found that the group that was most distant from the set of reference varieties was the western group, which primarily comprised Galician and Extremadura accessions.

Accessions originating from the same population classified in different RPPs indicated a high likelihood of contamination. This could be attributed to the natural hybridization

occurring within the propagation fields. Alternatively, it could be associated with a less rigorous selection of seeds [68], citing Brown's description [69], which characterized "landraces as geographically or ecologically distinctive populations, which exhibit conspicuous diversity in their genetic composition, both inter-population [i.e., between landraces, ACZ] and intra-population". Such a phenomenon could potentially occur within the Galician populations, from which the Galician commercial cultivars 'Caaveiro' and 'Callobre' were selected (classified as RPP2a by the Structure software).

The Galician ecotypes assessed in this study did not possess local names and could not be associated with those documented by Gadea [14] and Sanchez-Monge [15]. Consequently, they should not be classified as landraces in accordance with the definition provided by Villa et al. [42]. This definition posits that "a farmer's population may constitute an ecotype, and a collection of ecotypes cultivated in a specific region (such as a village), possessing a local name, will be classified as a landrace" (Chorlton, personal communication). However, these ecotypes align with the concept of a landrace as proposed by Villa et al. [42], which does not incorporate the local name in the definition proposed as follows: "A landrace is a dynamic population(s) of a cultivated plant that has a historical origin, a distinct identity, and lacks formal crop improvement, while often being genetically diverse, locally adapted, and associated with traditional farming systems".

In the context of wheat landraces, a considerable degree of genetic diversity has been observed among specimens collected across varying time frames, geographical regions, and climatic zones. Consequently, the genetic variability inherent in these landraces can be influenced by a multitude of factors. These include the specific years of collection, the anthropogenic impact exerted through dynamic storage practices, a range of needs and end-uses, the exchange of seeds (which facilitates gene flow) between farmers, and the geographical and environmental conditions prevalent in the regions where the wheat is cultivated [65].

Within the scope of our research, a number of ecotypes have evolved into cultivars such as the registered commercial cultivars 'Caaveiro', 'Callobre' and 'Castrexo' (E16L30) as well as the elite line (1910-4). Each of these local cultivars exhibited a unique genotype after selection. However, the elite lines E1L2 (registered as cultivar 'Miño') and 1967-5 (registered as cultivar 'Arzua') demonstrated variation for seven and one SSR, respectively. Despite this variability, the stability of the agromorphological characteristics was consistent with the registration requirements.

Structure and NJ tree analyses with the 156 wheat genotypes revealed that 2 ecotypes from Lugo (41 and 43) were grouped, albeit not mixed, with commercial cultivars. This suggests greater genetic proximity to these cultivars compared to other ecotypes and local cultivars, which were the only ones that approximated the height of commercial cultivars (Urquijo, personal communication). Ecotypes 41 and 43 have a similar height and date of flowering to other ecotypes collected in proximate areas from Lugo, such as the ecotypes 97 and 57 (Folgoso do Caurel), 110 (As Nogais), and 146 (Monforte de Lemos), which were tested from 2012 to 2014 [17]. However, these were not incorporated in this study involving SSRs.

Galician ecotypes 41 and 43 align with the explanation provided by Villa et al. [42] regarding the belief that "landraces can even be selected from cultivars". Terms such as 'creolization' and 'rustication' are employed in this context. As Zeven [70] elucidated, "in the absence of traditional and formal maintenance breeding, any improved landrace (cultivar), including a hybrid variety, will regress with time into a landrace". Furthermore, as per Ambrose's personal communication, "a cultivar that has been growing under a low selection pressure for specific traits but not uniformity for a long time could be considered a landrace". In the northwestern region of Spain, commercial wheat cultivars have predom-

inantly been cultivated since the 1980s. These could have undergone an ancient process of rustication due to the abandonment of rural areas or the transition of crops to pastures and forests, particularly in isolated valleys, such as O Caurel, which is the origin of ecotypes 41 and 43. Conversely, it is plausible that these ecotypes, in comparison to the rest of the Galician wheat ecotypes, have been introduced from another Spanish region, mirroring the background of the commercial cultivars.

4.3. Traceability of Galician Wheat in Flour, Sourdough and Bread Samples

Traceability constitutes a primary concern in the realm of quality brands, particularly in PGIs such as 'Galician Bread'. Molecular markers have proven to be robust tools in tracing the journey of Galician wheat from the field to the table, predominantly in the case of flours and sourdoughs, and are aligned with other techniques such as microscopy and neuronal networks [40,41]. However, their efficacy is somewhat diminished in bread due to DNA fragmentation during the baking process [71]. Our study affirmed the utility of a set of 17 SSRs in tracing the lineage of Galician wheat. This set demonstrated previously positive results with 'Caaveiro' [38] and was applied in this study to a comprehensive sample of Galician wheat, flour, sourdough, and bread. In the 58 samples of flours, sourdoughs, and breads analyzed in this study, 27.6% were found to be mislabeled. This finding underscores the efficacy of this set of SSRs in identifying fraud or contamination during the baking process.

Some other studies have addressed the traceability of specific wheats in seed blends and transformed products such as semolina, bread, and pasta. A previous study of traceability with ddPCR identified and quantified MTW seeds mixed with a midge-susceptible cultivar at a 9:1 ratio [20]. In a separate investigation, Morcia et al. [21] employed a cdPCR assay to accurately quantify a 3% contamination level of wheat within the pasta production chain, encompassing the stages from raw materials to the final products derived from durum wheat (*Triticum durum*). SSRs have been used for the detection of common wheat in semolina and breads using qualitative PCR [22], with detection thresholds of 3 and 5%, respectively, which was later reduced to 2.5% through the application of real-time PCR for monitoring the production of Altamura bread, a European Protected Designation of Origin (PDO) mark. Later, Morcia et al. [21] used successful digital PCR for genotype quantification in a pasta production chain. Sonnante et al. [24] and Carloni et al. [25] used real-time PCR to assess the exclusive use of durum wheat in pasta and traditional durum wheat breads.

Similar to our study, Fanelli et al. [26] evaluated SSRs to identify the durum wheat varieties in the production of semolina and commercial pasta, tracing the use of the varieties declared by the producer in comparison with other durum wheat cultivars commonly used in pasta production. They found foreign alleles, suggesting potential cross-contamination. Furthermore, they assessed the accuracy of the blend, with a detection limit of 5% (*w*/*w*).

5. Conclusions

Molecular markers were instrumental in identifying cross-pollination within CIAM-conserved ecotypes, facilitating their homogenization for local cultivar certification. These markers also confirmed their utility in tracing genotypes and their derived products, particularly the local flour used in high-quality Galician bread production. In this study, we identified a misclassification rate of 27.6% among the analyzed samples of flours, sourdoughs, and breads. Galician wheat exhibited higher diversity compared to the tested modern cultivars, representing a distinct gene pool crucial for maintaining Galician bread quality in breeding programs. The specific alleles of Galician wheat enable effective traceability in Galician bread production under the PGI designation, particularly in flour

and sourdough. However, its application in baked breads is limited due to the difficulty in obtaining PCR amplification fragments after baking.

This molecular approach enhances the authenticity guarantees of the PGI European mark and contributes to improved preservation strategies for the local wheat collection housed in the CIAM-AGACAL germplasm bank. These findings underscore the importance of conserving and utilizing Galician wheat genetic resources to maintain the unique characteristics of traditional Galician bread.

As a practical conclusion, we have developed a database employing 17 SSRs to trace the use of Galician wheat from farm to table, ensuring the authenticity of the PGI "Galician bread".

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15010051/s1, Figure S1: Delta K resulting from a Bayesian analysis with Structure software [45] on 17 SSRs applied to wheat ecotypes and cultivars (a) and wheat ecotypes and cultivars excluding commercial varieties (b); Table S1: Accessions classified according to the reconstructed population resulting from a Bayesian analysis using Structure software [45] on 17 SSRs applied to wheat ecotypes and cultivars, and to wheat ecotypes and cultivars excluding commercial varieties; Table S2: Commercial varieties and local ecotypes and cultivars of wheat evaluated with 17 SSRs. Sample Size, No. Alleles, No. Effective Alleles, Information Index, Observed Heterozygosity, Expected and Unbiased Expected Heterozygosity, and Fixation Index; Table S3: Exclusive alleles for commercial cultivars and for local cultivars and ecotypes. Factorial component analysis (FCA) was performed using the principal components (PC) method in SPSS V.28.

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