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Genetic diversity analysis of the durum wheat Graziella Ra, *Triticum turgidum* L. subsp. *durum* (Desf.) Husn. (Poales, Poaceae)

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ABSTRACT For the first time, the durum wheat Graziella Ra was compared to four Italian durum wheat varieties (Cappelli, Grazia, Flaminio and Svevo) and to Kamut in order to preliminary characterize its genome and to investigate genetic diversity among and within the accessions by Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) and α-gliadin gene sequence analysis. The main aim of the study was an attempt to determine the relationship between the historic accession Graziella Ra and Kamut which is considered an ancient relative of the durum subspecies. In addition, nutritional factors of Graziella Ra were reported. Obtained results showed that (i) both AFLP and SSR molecular markers detected highly congruent patterns of genetic diversity among the accessions showing nearly similar efficiency; (ii) for AFLPs, percentage of polymorphic loci within accession ranged from 6.57% to 19.71% (mean 12.77%) and, for SSRs, from 0% to 57.14% (mean 28.57%); (iii) principal component analysis (PCA) of genetic distance among accessions showed the first two axes accounting for 58.03% (for AFLPs) and 61.60% (for SSRs) of the total variability; (iv) for AFLPs, molecular variance was partitioned into 80% (variance among accessions) and 20% (within accession) and, for SSRs, into 73% (variance among accessions) and 27% (within accession); (v) cluster analysis of AFLP and SSR datasets displayed Graziella Ra and Kamut into the same cluster; and (vi) molecular comparison of α-gliadin gene sequences showed Graziella Ra and Kamut in separate clusters. All these findings indicate that Graziella Ra, although being very similar to Kamut, at least in the little part of the genome herein investigated by molecular markers, may be considered a distinct accession showing appreciable levels of genetic diversity and medium-high nutritional qualities.

KEY WORDS AFLP, α-gliadin gene, durum wheat; genetic diversity analysis, nutritional qualities, SSR; *Triticum.*

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INTRODUCTION

Durum wheat (*Triticum turgidum* L. subsp. *durum*) is the only tetraploid (AABB, 2n=4x=28) species of wheat of commercial importance that is widely cultivated today. It originated thousands of years ago from a hybridization (pollen exchange) of the wild diploid *T. monococcum* L. (A genome) and the donor of the B genome which, according to morphological, geographical and cytological evidence, has recently been recognized as *T. speltoides* (Tausch) Gren. or a closely related species (von Buren, 2001). In the last decades, a huge number of durum wheat cultivars have been obtained by artificial selection, generally based on high yield, disease resistance and technological qualities (e.g. bread- or pasta-making qualities) with little emphasis on taste or dietary components. On the other hand, at the same time, traditional local varieties have been considerably reduced as a result of the diffusion of new varieties of wheat. To preserve genetic variability and reduce genetic erosion it is extremely important developing and maintaining local collections, including old cultivars and landraces, which – at least in some cases – may be employed for niche cereal-based typical products. This was the case of Graziella Ra, an ancient accession (not a cultivar) of durum wheat which, thanks to its

good taste and fine pasta-making qualities, recently appeared on the market as Graziella Ra®, an Italian trademark used in marketing products made with the homonymous grain. Currently, it is organically grown in Marches (central Italy) by Alce Nero Cooperative (Urbino, PU) mainly with the aims to contribute to the preservation of local biodiversity and increase the interest for ancient crops which are at the basis of the Mediterranean diet.

This study was designed with the intent of providing a preliminary characterization of Graziella Ra genome, analysed for the first time. To this aim, other five accessions chosen as representatives of modern (Grazia, Flaminio, Svevo), traditional (Cappelli) and ancient (Graziella Ra, Kamut) wheats were selected to obtain a small set of three modern and three older durum accessions. Comparative analysis was carried out by AFLPs (Amplified Fragment Length Polymorphisms), microsatellites (SSRs, Simple Sequence Repeats) and the α-gliadin gene sequence to evaluate genetic diversity within and among wheats under study.

MATERIALS AND METHODS

Accessions

Graziella Ra (Fig. 1a) is a type of durum wheat characterized by low yield (15-20 quintals per hectare), medium-long cycle, tall size (about 120 cm) and a phenotype very similar to Kamut's (see below) with large ears and long aristas. It was brought to Italy at the end of '70s (see http://www.alcenerocooperativa.it/pagina.asp?pag=443), forgotten for a long time and rediscovered a few years ago due to its fine pasta-making qualities. Cappelli is an Italian traditional strain of durum wheat which deserves a privileged place among the varieties of old established durum wheat for being the very first selected variety. Svevo, Grazia and Flaminio are modern cultivars, with a great commercial importance, employed for pasta or bread-making. Kamut is a registered trademark of Kamut International, Ltd., used in marketing products made with the variety QK-77. It is characterized by erect young shoots with very narrow pubescent leaves, the plants tiller very little and the straw thin. The spikes are narrow, lax or very lax with long narrow white glumes. The spikelet lemmas have long and strong, more or less deciduous, white or black awns (Fig. 1b). The grains are very large - up to twice the size of bread wheat kernels - narrow, vitreous, and flinty with a characteristic hump. The correct subspecies is still in dispute; in fact, according to Stallknecht et al. (1996), Kamut has been classified, from time to time, as *T. turgidum polonicum*, *T. turgidum turanicum* or *T. turgidum durum*. Although its taxonomy is contentious, it is considered an ancient relative of durum subspecies. All wheats were provided by Alce Nero Cooperative, with the exception of Kamut, kindly supplied by Molini del Conero (Osimo, AN, Italy).

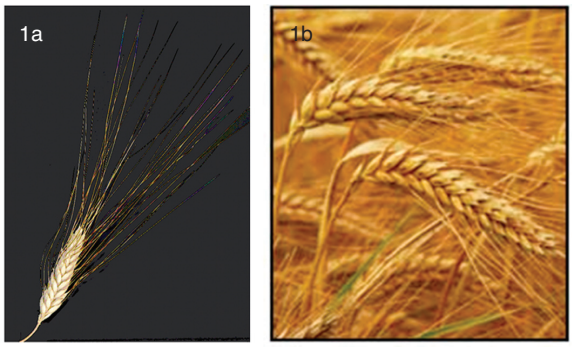

Figure 1. Spike morphology of Graziella Ra (1a) and Kamut (1b) durum wheats.

DNA extraction

Several seeds of each line were germinated in the dark for two days. The seedlings were grown in daylight for seven days. Leaf tissues – sampled at the four-leaf stage from twenty different plants per accession – were immediately frozen in liquid nitrogen and ground in a mortar with a pestle. Thirty mg of powder were used for DNA extraction following the cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1990) with slight modifications. DNA quality was tested by a 0.8% agarose gel electrophoresis.

AFLP

AFLP genotyping was performed at Keygene NV (Wageningen, The Nertherlands) using their standard in-house developed protocols (Vos et al., 1995). Briefly, DNA extracted from four different plants for each parental line (for a total

of twenty-four samples) was fingerprinted using ten AFLP primers, five *Pst*I (indicated as P35, P36, P39, P41, P42) and five *Taq*I (T40, T41, T42, T44, T46) (Table 1) arranged in eight primer combinations (P35/T44; P35/T46; P36/T46; P39/T41; P39/T42; P41/T40; P41/T41 and P42/T41) (Table 2).

Microsatellite genotyping

Twelve different plants per accession (seventy-two individuals) were employed. Nine Simple Sequence Repeat (SSR) markers were selected from several ones tested on the grounds of their Tm, length and degree of polymorphism. Primers are listed in Table 3. A tailed PCR primer was used for SSR analysis by adding a 19-base M13 oligo sequence (M13 tail) to the 5′ end of each forward SSR primer. Thus, each SSR reaction used three primers: two unlabelled SSR primers one of which having an attached M13 sequence tail (5'-CACGACGTTGTAAAACG AC-3'), and one universal FAM-labelled M13 primer with the same sequence as the M13 tail (Schuelke, 2000; Boutin-Ganache et al., 2001; Fukatsu et al., 2005). PCR reactions were carried out in 10 µl of a solution containing 10 ng genomic DNA, 1x Mg-free PCR buffer solution, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 50 nM forward primer, 5.0 nM reverse primer, 500 nM M13-labelled primer, 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and nuclease-free water. Amplification was performed as follows: 5 min at 95 °C; 20 sec at 94 °C, 30 sec at 55 °C, 30 sec at 72 °C (42 cycles); and a final extension stage of 5 min at 72 °C. PCR products were separated with an ABI 3730 DNA sequencer (Applied Biosystems) and the fragments were sized by means of a ladder labelled with a fluorochrome VIZ (LIZ500, Applied Biosystems). Data were analysed with GeneMapper 3.0 (Applied Biosystems).

A-gliadin gene Amplification, Cloning, Sequencing and Analysis

DNA from two plants per accession (total of twelve samples, different from samples employed for molecular markers) was used for PCR amplifications of the α-gliadin gene. Both forward (5'–ATGAAGACCTTTCTCATCC–3') and reverse (5'–YYAGTTRGTACCGAAGATG CC–3') primers were designed on the conserved 5' and 3' ends of the coding region of the α-gliadin gene sequences downloaded from the GenBank database (ID: DQ296195, DQ296196 and AJ870965). PCR amplifications were carried out - using a high fidelity Pfu DNA Polymerase (Promega) - as follows: 95 °C for 2 min; 95 °C for 1 min, 60 °C for 30 sec, 72 °C for 2 min (30 cycles); 72 °C for 5 min. Reaction products were visualized by electrophoresis on a 1.2% agarose gel containing TBE 1X buffer and ethidium bromide (0.5 µg/ml). An aliquot (1 µl) of the PCR product was inserted into a pCR 4-TOPO vector by the TA-cloning system and transformation was performed on *E. coli* TOP10 cells following the manufacturer's instructions (Invitrogen). Selected transformants were analysed for presence of the insert by PCR, grown in LB medium overnight and purified by the Wizard Plus SV minipreps kit (Promega). Finally, sequencing of plasmid inserts was done by using automated DNA sequencers at Eurofins MWG Operon. Sequences were visualized with BioEdit Sequence Alignment Editor 7 (Hall, 1999), aligned with the ClustalW option included in this software and double checked by eye. Standard measures of nucleotide polymorphism [mean pairwise differences (k), nucleotide diversity (π = Pi and π_{JC} = Pi corrected according to Jukes and Cantor) and nucleotide divergence (D_{xy}) between accessions] using the full set of all sequences were computed by DNAsp 5 (Librado & Rozas, 2009).

Statistical analysis

For AFLP and SSR datasets, analyses were performed within GenAlEx 6.4 (Peakall & Smouse, 2006), a user-friendly package with an intuitive and consistent interface that allows to analyse a wide range of population genetic data, including both dominant (AFLP) and codominant (SSR) datasets, within MS Excel. For each accession, allele number (Na), heterozygosity (He), number and frequency of genotypes and percentages of polymorphic loci were obtained by the software. Polymorphism information content (PIC) of each SSR was computed according to Botstein et al. (1980). Nei's unbiased genetic distance (Nei, 1978) was

Primer name	*sequence*
P35	5'-GACTGCGTACATGCAG ACA-3'
P36	5'-GACTGCGTACATGCAG ACC-3'
P39	5'-GACTGCGTACATGCAG AGA-3'
P41	5'-GACTGCGTACATGCAG AGG-3'
P42	5'-GACTGCGTACATGCAG AGT-3'
T40	5'-GATGAGTCCTGACCGA AGC-3'
T41	5'-GATGAGTCCTGACCGA AGG-3'
T42	5'-GATGAGTCCTGACCGA AGT-3'
T44	5'-GATGAGTCCTGACCGA ATC-3'
T46	5-GATGAGTCCTGACCGA ATT-3'

Table 1. *Pst*I (P) and *Taq*I (T) primers employed for AFLP analysis

Primer combinations	No. of polymorphic bands	Mean diversity index (He)	Marker index*
P35/T44	19	0.082	1.56
P35/T46	11	0.018	0.20
P36/T46	16	0.087	1.39
P39/T41	23	0.044	1.01
P39/T42	13	0.003	0.04
P41/T40	17	0.010	0.17
P41/T41	13	0.029	0.38
P42/T41	25	0.032	0.80

*MI = (no. of polymorphic loci/PC) x (mean diversity index/PC); for details, see Powell et al. (1996).

Table 2. Polymorphism features of the eight AFLP primer combinations (PCs) used to estimate genetic similarities among wheat accessions under study.

SSR	*Primer sequence*
Barc174	For 5' - TGGCATTTTTCTAGCACCAATACAT Rev 5' - GCGAACTGGACCAGCCTTCTATCTGTTC
DuPw217	For 5' -CGAATTACACTTCCTTCTTCCG Rev 5' -CGAGCGTGTCTAACAAGTGC
Xgwm750	For 5' - CTTGCACAGAGACGATGCAT Rev 5'-TGAGTCAGTCTCACAACCGG
Xgwm1045	For 5' - ATCACAAGGAGTTTATCGCT Rev 5'- GTCAATGGACCATGGGATTC
Xgwm1038	For 5' - GTGCTCCATGGCGTCTG Rev 5' - AGTCCAGCAAACATTCTCCA
Xgwm126	For 5' - CACACGCTCCACCATGAC Rev 5' - GTTGAGTTGATGCGGGAGG
Xgwm1027	For 5' - CAGTTCTCCCGGCATGTATT Rev 5' - TTCACATTGTCGCGTTGAAT

Table 3. List of primers used for SSR analysis

calculated in the TFPGA program (Miller, 1997). For both AFLPs and SSRs, phenetic diagrams were constructed on corresponding pairwise genetic distance matrices by the Unweighted Pair-Group Method using Arithmetic averages (UPGMA) (Sneath & Sokal, 1973) with the UPGMA tree searching algorithm of the software. A thousand replicate distance matrices were bootstrapped to evaluate the robustness of the trees. For both datasets, analysis of molecular variance (AMOVA) was carried out to examine total genetic variation among and within accessions; in addition, Principal Component Analysis (PCA) was performed in order to more effectively view the patterns of genetic distance. A Mantel test was used to detect the possible correlation between AFLP and SSR accession matrices. Statistical significance was determined by random permutations, with the number of permutations set to 9,999.

Phylogenetic analysis

Phylogenetic analyses were conducted in MEGA 5 (Tamura et al., 2011) and BEAST 1.4.8 (Drummond & Rambaut, 2007) by Maximum Likelihood (ML) and Bayesian Inference (BI). For maximum likelihood analyses, the most appropriate model of DNA substitution resulted HKY (Hasegawa Kishino Yano). Bayesian analysis was conducted by BEAST where the topology and divergence times can be estimated simultaneously from the data and therefore a starting tree topology is not required, making it particularly appropriate for groups with uncertain phylogenies. BEAST input files were generated with BEAUTi (v 1.4.8) using the α-gliadin gene dataset (nexus format) and a HKY substitution model. For partition into codon positions, the SRD06 model (Shapiro et al., 2006) was selected; this model links 1st and 2nd codon positions but allows the 3rd positions to have a different relative rate of substitution, transition-transversion ratio and gamma distributed rate heterogeneity and has been found to provide a better fit for protein-coding nucleotide data. BEAST was run for 1,000,000 generations with samples taken every 100 generations. Five independent Markov Chain Monte Carlo (MCMC) runs were conducted and the log and tree files were combined using LogCombiner (v 1.4.8). The results were examined by Tracer (v 1.5) to confirm stationary distribution and adequate effective sample sizes (i.e. ESS>200) for all parameters, indicating that the sampled generations were uncorrelated and the posterior distribution of the parameter was long and accurate. TreeAnnotator (v 1.4.8) was then used to summarize a best supported tree and annotate the tree with posterior probabilities of the nodes under investigation. FigTree (v 1.3.1) was used to display the 95% confidence intervals. BEAST, BEAUTi, LogCombiner, Tracer, TreeAnnotator and FigTree were downloaded from http://beast.bio.edu.ac.uk.

Support for the internodes was assessed by bootstrap percentages (100 replicates for ML), whereas for Bayesian inference tree, the Bayesian posterior probability was computed. A-gliadin gene sequences from *Triticum aestivum* L. (GenBank ID: DQ166377) and *T. dicoccoides* Korn. (GenBank ID: DQ140352) were employed as outgroups.

Nutritional quality

Graziella Ra was investigated by Eurofins Biolab srl (an Italian company specialized in assays and controls, and in biological, microbiological and chemical determinations) using their standard in-house developed protocols; each analysis was made in triplicates. For Kamut, we report nutritional values available at http://www.kamut.com.

RESULTS AND DISCUSSION

Molecular marker variation

A total of twenty-four individuals were investigated using eight AFLP primer combinations. One sample (from Svevo) didn't generate reliable fingerprintings and was excluded from the analysis which, therefore, resulted in twenty-three individuals showing a total of 137 markers. For each AFLP primer combination, number of polymorphic bands, mean heterozygosity and marker index are reported in Table 2. The presence/absence of each fragment was encoded as a 1/0 score, generating a binary data matrix. Within each accession, mean heterozygosity ± standard error

SSR	Chromosome	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	PIC	Mean He	MI*
Xgwm126	5A	203	206	212	214	-		0.60	0.111	0.44
Barc174	1B	200	201	216	-	-		0.45	0.030	0.09
Xgwm1045	2A	192	198	202	-	-		0.61	0.058	0.17
Xgwm1038	3A	235	241	242	252	254	274	0.35	0.072	0.43
Xgwm750	1A	230	234	236	249	-		0.68	0.102	0.41
Xgwm1027	2B	125	129	138	-	-		0.67	0.074	0.22
DuPw217	6B	232	241	242	-	-		0.30	0.025	0.07

MI = (no. of polymorphic bands/SSR) x (mean diversity index/SSR); for details see Powell et al. (1996).

Table 4. List of all the alleles revealed by the microsatellites in the six accessions. Chromosome mapping, PIC, Polymorphism Information Content; He, heterozygosity (also called diversity index); MI, Marker index are reported. Please note that alleles are expressed in nucleotide length (bp = base pairs).

and percentage of polymorphism resulted specifically: 0.024±0.008, 6,57% (Svevo); 0.081±0.014, 22.63% (Flaminio); 0.027±0.008, 8.03% (Kamut); 0.051±0.012, 11.68% (Graziella Ra); 0.034±0.010, 8.03% (Cappelli); and 0.083±0.015, 19.71% (Grazia). Percentage of polymorphism was, on average, 12.77%.

SSR data were classified according to a qualitative scale, with scores ranging from 1 to 5, describing the complexity of the amplification profile for each primer (Stephenson et al., 1998). Out of nine markers considered, seven [Barc174, Xgwm750, Xgwm1038, Xgwm126 and Xgwm1027 (score 1, 2); Xgwm1045 and DuPw217 (score 3)] were included in the analysis; whereas two (Xgwm1136 and Xgwm1009) failed to give rise to any amplification products. SSRs revealed a total of 26 alleles in the six accessions. The number of alleles per locus varied among these markers, ranging from three (DuPw217, Barc174, Xgwm1027, Xgwm1045) to six (Xgwm1038) with an average of 3.7. As a measure of the informativeness of microsatellites, the average PIC (Polymorphism Information Content) value was 0.53, ranging from 0.30 (DuPw217) to 0.68 (Xgwm750). For each marker, number of alleles, PIC value, mean heterozygosity and marker index (MI), a universal metric to represent the amount of information obtained per experiment, are reported in Table 4. As shown, marker index values are not very high but, on the other hand, considering that a *PIC* value > 0.5 accounts for a highly informative marker, 0.5 > *PIC* > 0.25 for an informative marker, and PI*C* ≤ 0.25 for a slightly informative marker (Botstein et al., 1980), PIC values suggest that SSRs employed in the present study resulted adequate and efficient. With reference to the percentage of polymorphism within each accession, observed values ranged between 0% (Kamut) and 57.14% (Svevo and Graziella Ra), going through 14.29% (Flaminio and Grazia) and 28.87% (Cappelli), with an average value of 28.57%. Based on SSR markers herein reported along with the limited number of accessions under investigation, Table 5 summarizes private alleles observed in this study, which may be used as a simple indirect measure

Accession	*SSR*	*Allele (in bp)*	*Freq (%)*
Svevo	Xgwm126	203	66.7
Svevo	Xgwm126	212	16.7
Svevo	**Xgwm1038**	**235**	**4.2**
Svevo	Xgwm1038	252	95.8
Flaminio	Barc174	216	10
Graziella Ra	Xgwm750	249	9.1
Cappelli	Xgwm750	236	33.3
Cappelli	DuPw217	242	8.3
Grazia	**Xgwm1038**	**241**	**4.2**
Grazia	Xgwm1038	254	12.5
Grazia	**Xgwm1038**	**274**	**4.2**
Grazia	**DuPw217**	**232**	**100**

Table 5. Unique alleles observed by SSR molecular markers. Rare (frequency < 5%) and diagnostic alleles (frequency = 100%) are in bold.

of genetic diversity. As shown, Grazia and Svevo have the greatest number (four) of accession-specific alleles; moreover, in Grazia the 232 bp allele is monomorphic and hence could be considered as diagnostic for the identification of the variety; private alleles were observed in nearly all the accessions, though three of them were rare, with a percentage below 5%.

Average heterozygosities for AFLPs and SSRs were not significantly different (*t* test, $p > 0.05$).

Cluster analysis

Genetic distance was calculated using Nei's index. Cluster analysis applied to genetic distance matrices produced the phenetic diagrams shown in figures 2a and 2b. In both cases, Kamut and Graziella Ra resulted very similar.

For both AFLPs and SSRs, patterns of PCA revealed by the first two principal coordinate axes accounted for the most of the variation in the data, and so only the first two dimensions were plotted in this paper. With reference to pairwise individual genetic distance matrices, the first two axes accounted for 63.15% (38.56% and 24.59%) of the AFLPs and 64.42% (42.26% and 22.16%) of the SSRs variation (Figs. 3a and 3b); taking into account PCA of genetic distances among accessions, the first two axes explained 58.03% (34.95% and 23.08%) of the AFLPs and 61.60% (36.11% and 25.49%) of the SSRs variation. As shown in figures 3c and 3d, a high degree of similarity between Graziella Ra and Kamut was confirmed also by PCA.

Analysis of molecular variance (AMOVA)

Analysis of molecular variance partitioned the total genetic variance into variance among populations and within population. For AFLPs, total variance was partitioned into 80% (variance among populations) and 20% (within population) (Fig. 4a); for SSRs, into 73% (among populations) and 27% (within population) (Fig. 4b).

Correlation between AFLPs and SSRs

A strong correlation ($r^2 = 0.92$) between AFLP and SSR population data matrices was obtained by the Mantel test (Fig. 5). This finding suggests that both types of molecular markers detected highly congruent patterns of genetic diversity, at the accession level, showing nearly similar efficiency. In fact, AFLP and SSR average heterozygosities were not significantly different and observed values of MI or polymorphism levels were in line with distinctive nature of these markers. In particular, a higher MI for AFLPs (0.69 *vs.* 0.26) was the result of a higher multiplex ratio component, due to the simultaneous detection of several polymorphic markers per single reaction. On the contrary, a lower number of total bands was obtained for SSRs, but all of these were polymorphic, thus giving a higher average percentage of polymorphism (28.57% *vs.* 12.77%) and providing higher genetic diversity within a given accession and lower genetic differentiation among accessions than AFLP markers, which was confirmed by AMOVA results as well.

A-gliadin gene

A-gliadin is a very important storage protein widely studied for its implication in coeliac disease (i.e. Koning, 2005; Gregorini et al., 2009 and references therein). In this study molecular analysis of the α- gliadin gene sequence was employed either to analyse diversity at the gene level or to provide a possible reconstruction of phylogenetic relationships among wheats under study.

A-gliadin gene complete sequences obtained in this study are available at GenBank as GQ999807 (Cappelli, 903 bp), GQ999809 (Flaminio, 942 bp), GQ999811 (Grazia, 963 bp), GQ999813 (Graziella Ra, 909 bp), GQ999815 (Kamut, 942 bp), GQ999817 (Svevo, 942 bp). Sequences alignment showed 78 variable sites, 79 mutations (S = 78, Eta = 79) and 75 insertions/deletions. Nucleotide diversity (π) was 0.032 ± 0.011 and 0.033 when corrected according to Jukes and Cantor (π_{JC}). The average number of nucleotide differences (k) was 28.867. Assessed mean sequence identity was 91.5%; in particular, α-gliadin genes from Graziella Ra and Kamut were 95% identical. Deduced α-gliadin protein sequences showed a mean identity of 89.4%; α-gliadins from Graziella Ra and Kamut were 94.3% identical. Maximum likelihood and Bayesian Inference phylogenetic reconstructions produced nearly identical results. ML and BI

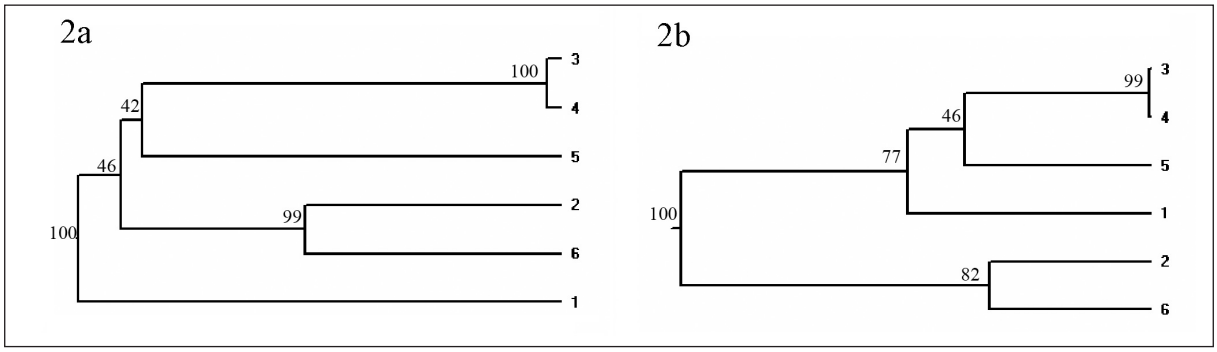

Figure 2. 2a. Dendrograms of the six wheat accessions based on Nei's genetic distance calculated using 137 amplified fragment length polymorphisms (AFLPs); 2b. Dendrograms of the six wheat accessions based on Nei's genetic distance calculated using seven simple sequence repeats (SSRs). 1, Svevo; 2, Flaminio; 3, Kamut; 4, Graziella Ra; 5, Cappelli; 6, Grazia. Bootstrap supporting values (1,000 replicates) are reported on the nodes.

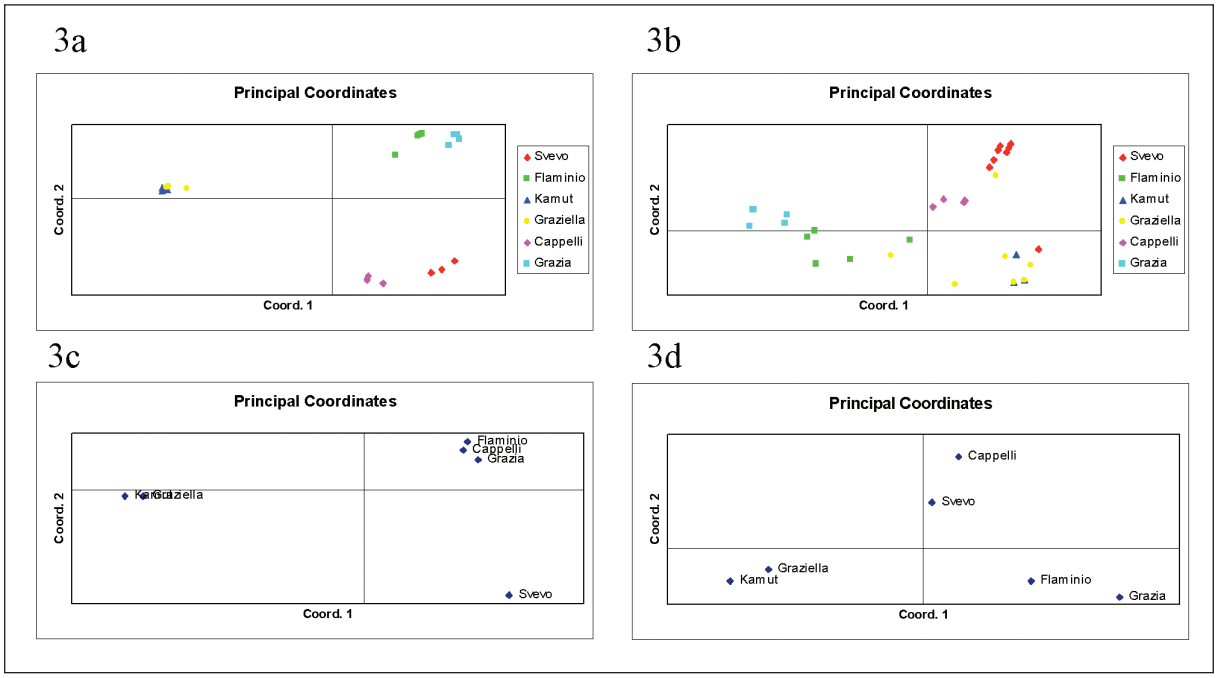

Figure 3. Principal Component Analysis (PCA) plots of the first two axes based on genetic distance matrices among individuals for AFLP (3a) and SSR (3b) datasets; PCA plots of the first two axes based on genetic distance matrices among accessions for AFLP (3c) and SSR (3d) datasets.

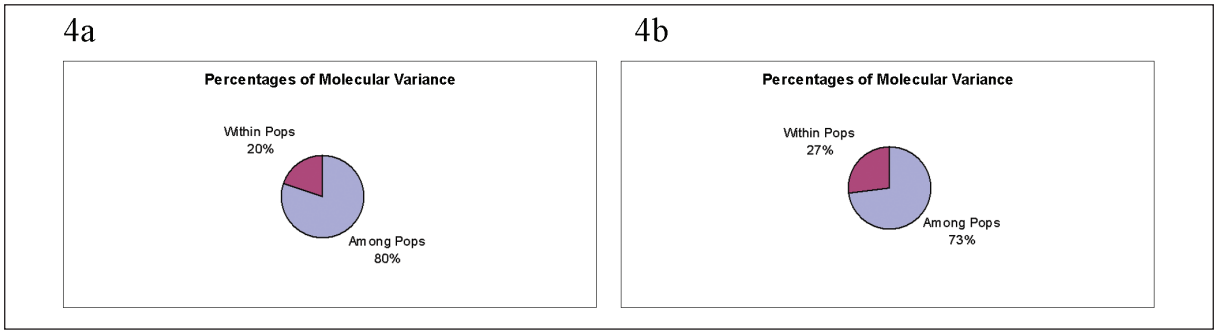

Figure 4. Results of Analysis of Molecular variance (AMOVA) for the total AFLPs (4a) and SSRs (4b) showing the percentage of variation among and within accessions.

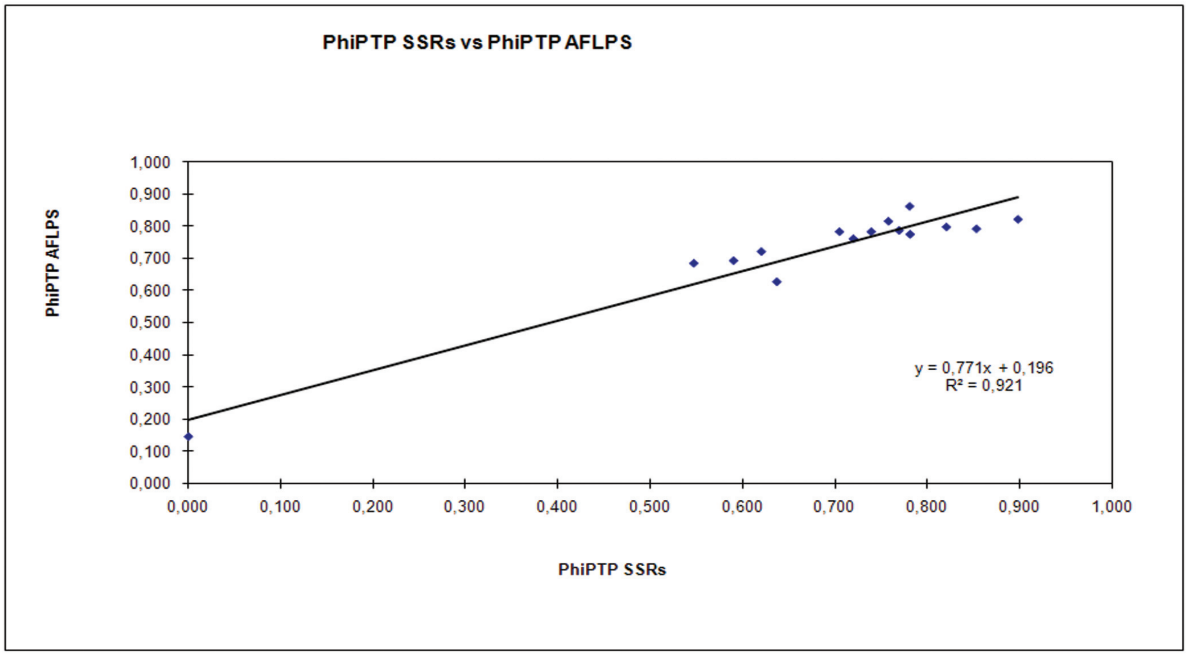

Figure 5. Output of Mantel test comparing the AFLP and SSR genetic distance matrices at accession level.

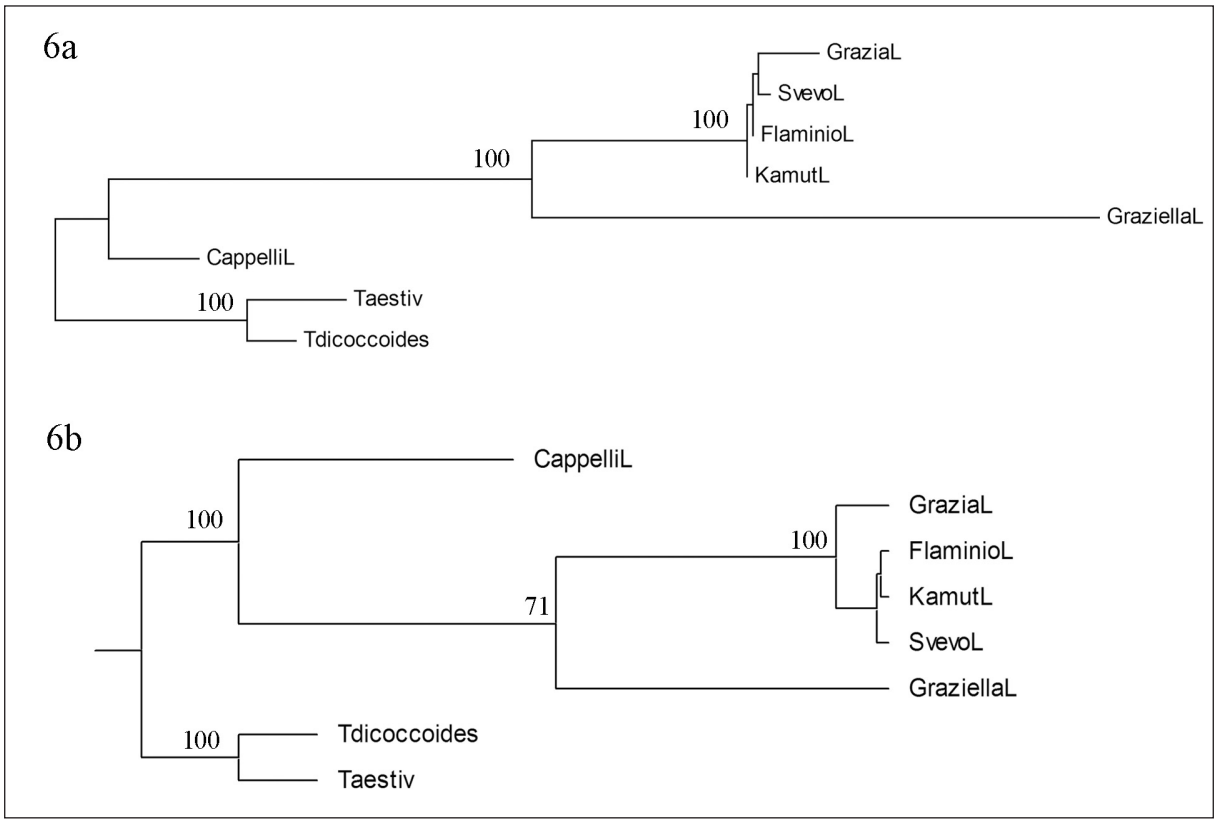

Figure 6. 6a. 50% majority rule Maximum Likelihood consensus tree inferred from the α-gliadin gene sequence alignment. Numbers above branches represent bootstrap values (100 replicates). 6b. Bayesian consensus tree inferred from the α-gliadin gene sequence alignment. Numbers above branches represent Bayesian posterior probabilities. *T. aestivum* and *T. dicoccoides* were employed as outgroups to root the trees.

	*Common wheat**	*Kamut*	*Graziella Ra*
Water	11.5%	9.8%	10.8%
Protein**	14%	19.6%	11.80%
Total lipid (fat)	1.9%	2.6%	2.91%
Carbohydrate	72.7%	68.2%	61.23%
Crude fiber	2.1%	1.8%	2.7%
Ash	1.66%	1.82%	2.02%
MINERALS (mg/100g)			
Calcium	30	31	31.2
Iron	3.9	4.2	2.5
Magnesium	117	153	85.3
Phosphorus	396	411	450
Potassium	400	446	379.1
Sodium	2.0	3.8	5.8
Zinc	3.2	4.3	38
Copper	0.44	0.46	0.5
Manganese	3.8	3.2	2.2
Selenium (mg/kg)		1.6-7	2
VITAMINS (mg/100g)			
Thiamine (B1)	0.42	0.45	>0.05
Riboflavin (B2)	0.11	0.12	0.02
Niacin	5.31	5.54	7.83
Panthotenic acid	0.91	0.23	0.04
Vitamin B6	0.35	0.08	0.94
Folacin	0.0405	0.0375	0.031
Vitamin E	1.2	1.7	0.43
AMINOACIDS (g/100g)			
Tryptophan	0.194	0.117	-
Threonine	0.403	0.540	0.42
Isoleucine	0.630	0.600	0.78
Leucine	0.964	1.23	0.86
Lysine	0.361	0.440	0.34
Methionine	0.222	0.250	-
Cystine	0.348	0.58	-
Phenylalanine	0.675	0.85	0.36
Tyrosine	0.404	0.430	0.21
Valine	0.624	0.800	0.46
Arginine	0.610	0.860	0.69
Histidine	0.321	0.430	0.29
Alanine	0.491	0.630	0.45
Aspartic acid	0.700	0.980	0.65
Glutamic acid	4.68	5.97	4.09
Glycine	0.560	0.650	0.47
Proline	1.50	1.44	1.31
Serine	0.662	0.930	0.51

* an average number for all the wheats in the USDA report was used; **European scale on dry matter

Table 6. Nutritional values for common wheat*, Kamut® brand wheat (both available at www. kamut.com) and Graziella Ra wheat (present paper).

consensus trees (Figs. 6a and 6b) showed that molecular clustering disagreed with morphological clustering, in fact, contrary to AFLPs and SSRs, phylogenetic analyses of α-gliadin gene sequences showed Graziella Ra and Kamut in separate clusters. This finding not only confirms that the two wheats are related but also supports the hypothesis that, although being similar – at least in the little part of the genome investigated by molecular markers employed in this study – Graziella Ra and Kamut may be considered distinct accessions.

Nutritional quality

Given that all parameters linked to nutritional qualities are affected by the environment and that we compared Graziella Ra (analysed in triplicates) with Kamut (whose nutritional quality is reported in the Kamut web site, without any descriptions of how each parameter was assessed) a real comparison (including statistics) was not possible. Nevertheless, it is noticeable that all values of dietary components of Graziella Ra are in line with mean values reported for Kamut and other commercially available durum wheats (Table 6). Hence, our results corroborate the idea that Graziella Ra may be considered an accession distinct from Kamut endowed by appreciable levels of genetic diversity and medium-high nutritional qualities.

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