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Genetic diversity in *Hordeum chilense* Roem. et Schult. germplasm collection as determined by endosperm storage proteins

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Abstract

Hordeum chilense Roem. et Schult. shows interesting characteristics for breeding, such as disease or pest resistance and has high variability for endosperm storage proteins, valuable for the improvement of the breadmaking quality of both tritordeum and wheat. Knowledge of the genetic structure and the level of *H. chilense* genetic diversity within its distribution zone may be important to decide on breeding strategies as well as management procedures. The pattern of genetic variation within and among different regions of the distribution area of *H. chilense*, was analysed by endosperm storage proteins (gliadin and low-molecular-weight glutenin subunits). Several analyses were performed including AMOVA, direct correlation between phenotypic and geographic distance matrices and spatial genetics differentiation. Most genetic diversity was caused by differences among individuals within a population, although all the analyses performed suggest the existence of a low degree of differentiation within regions. Correlation values between phenotypic and geographic distances were low but significant. The spatial genetics analysis revealed that the average phenotypic distances of each of the spatial distance class were not lower (or higher) than that expected by chance. These results are explained by their neutral behaviour towards a selection of endosperm storage proteins and could be very useful in optimising future sample collecting strategies.

Introduction

Hordeum chilense Roem. et Schult., native from Chile and Argentina, is a diploid wild barley included in the section *Anisolepis nevski*. This species is polymorphic both at the morphological and biochemical levels. *H. chilense* is a weak perennial much appreciated by cattle (Valderrama et al. 1991). The main interest of this species is based on its appreciable agronomic characteristics and on its high crossability with other members of the Triticeae tribe (von Bothmer and Jacobsen 1986; Martín et al. 1998). *H. chilense* has been reported to be resistant to different pests and diseases including *Schizapis graminum* (Castro et al. 1995), *Diuraphis noxia* (Clement and Lester 1990), *Fusarium culmorum* and *Septoria nodorum* (Rubiales et al. 1996) and *Septoria tritici* (Rubiales et al. 1992). In the same way, *H. chilense* shows benefits against abiotic stresses, being salt tolerant (Forster et al. 1990) and drought tolerant (Gallardo and Fereres 1989). In addition,

H. chilense shows a high variability for endosperm storage proteins valuable for breadmaking quality improvement (Atienza et al. 2000, 2002; Alvarez et al. 2001). Apart from these important agronomic characteristics, *H. chilense* has a high crossability with other members of the Triticeae tribe (*Aegilops*, *Agropyrum*, *Dasypirum*, *Hordeum*, *Secale*, *Triticum* and ×*Triticosecale* as reviewed by Martín et al. (1998)). In fact, from the crosses between *H. chilense* and diploid, tetraploid and hexaploid wheats originated fertile amphiploids, noted as Tritordeums (×*Tritordeum* Ascherson et Graebner). Hexaploid tritordeum ($2n = 6x = 42$, $\mathbf{AABBH^{ch}H^{ch}}$) shows promising characteristics as a new crop (Martín et al. 1999) having some potential for breadmaking (Alvarez et al. 1995; Alvarez and Martín 1996). The influence of endosperm storage proteins on breadmaking quality makes necessary the study of this component in both tritordeum and *H. chilense*: in fact some authors (Alvarez et al. 1993, 1999a) studied the endosperm storage proteins from hexaploid tritordeum using SDS–PAGE and acid–PAGE methods. Alvarez et al. (1999a) proved that the storage proteins synthesized by the $\mathbf{H^{ch}}$ genome influence the breadmaking quality of hexaploid tritordeum. Several studies showed the high variability for these proteins in both tritordeum (Alvarez et al. 1993, 1999a, b; Caballero et al. 2001) and *H. chilense* (Atienza et al. 2000, 2002; Alvarez et al. 2001). This variability is important for the improvement of breadmaking quality in tritordeum but it is also valuable in wheat using tritordeum as a genetic bridge. At present, new amphiploids have been generated using new and different accessions of *H. chilense* in order to increase the genetic basis of this novel cereal. In this work, most of the accessions used were collected in expeditions carried out by our group in the distribution area of the species (Tobes et al. 1995; Giménez et al. 1997). Consequently, the knowledge of the genetic diversity of *H. chilense* in its distribution zone is essential to maximise the utilization of this germplasm and for the design of correct sampling strategies.

The aim of this work is to get insight into the genetic distribution of endosperm storage proteins of *H. chilense* related with the distribution area of this species, to maximise the use of germplasm resources.

Material and methods

Plant material

Sixty-six lines of *H. chilense* collected from its complete distribution area were studied (Table 1). A homozygotic genotype was obtained from each line through two generations of self-pollination. Nine seeds from three different plants of each line were analysed to check the homogeneity of the progeny. The lines obtained are maintained at the Germplasm Bank of the Instituto de Agricultura Sostenible, Consejo Superior de Investigaciones Científicas (IAS, CSIC, Córdoba, Spain). All these lines were previously characterized for gliadins and glutenin composition (Atienza et al. 2000, 2002).

Protein extraction

Due to the small size of the *H. chilense* grain (≈3 mg), three embryoless seeds of each line were necessary to extract the endosperm storage proteins. Albumin and globulin fractions were removed with water and saline solution (0.5 M NaCl), respectively. The remaining sodium chloride was washed away with distilled water.

Monomeric prolamins were solubilized with 1 mL of ethanol (70%) and precipitated with cold acetone. The remaining cold acetone was evaporated off overnight at room temperature, and the dried pellet was solubilised in solution buffer (125 mM Tris–HCl (pH 6.8) + 2% (w/v) dithiothreitol + 0.005% bromophenol blue) in a ratio 1 : 10 (w : v) at 60 °C for 30 min. The tubes were stored at −20 °C.

Polymeric prolamins were extracted with a different buffer (125 mM Tris–HCl (pH 6.8) + 2% (w/v) dithiothreitol + 20% (v/v) glycerol + 0.005% (w/v) bromophenol blue) in a 1 : 10 ratio (w/v) at 60 °C for 30 min. The samples were centrifuged at 14 000 × *g* and the supernatant was transferred to a new tube and stored at −20 °C.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) separation

Gliadins and glutenins were separated in vertical SDS–PAGE slabs in a discontinuous Tris–HCl–SDS buffer system (pH 6.8/8.8) at a polyacrylamide

Table 1. Passport data of the lines of *H. chilense* Roem. et Schult. used in this study.

Line	Latitude	Longitude	Region[a]	Line	Latitude	Longitude	Region[a]
H7	30° 56′	71° 31′	1	H225	32° 18′	71° 31′	2
H8	34° 04′	70° 56′	3	H226	34° 03′	71° 38′	3
H16	32° 18′	71° 31′	2	H228	34° 04′	70° 56′	3
H17	30° 54′	72° 22′	1	H229	33° 38′	70° 18′	3
H35	34° 04′	70° 56′	3	H232	32° 25′	70° 55′	2
H39	34° 03′	71° 38′	3	H241	33°	70° 57′	3
H47	36° 45′	72° 18′	5	H245	34° 58′	70° 27′	4
H51	36° 45′	73° 09′	5	H250	38° 42′	73° 02′	6
H52	36° 45′	73° 09′	5	H251	38° 26′	71° 22′	6
H54	34° 51′	70° 34′	4	H252	38° 41′	73° 24′	6
H55	34° 51′	70° 34′	4	H254	34° 57′	70° 23′	4
H57	34° 45′	70° 34′	4	H255	38° 42′	73° 02′	6
H58	34° 45′	70° 34′	4	H261	30° 23′	70° 58′	1
H59	34° 45′	70° 34′	4	H266	30° 32′	71° 42′	1
H60	34° 45′	70° 34′	4	H283	30° 41′	70° 52′	1
H68	36° 45′	72° 18′	5	H286	29° 55′	71° 14′	1
H74	33° 21′	71° 23′	2	H292	30° 45′	71° 32′	1
H93	33° 06′	71° 28′	3	H293	30° 32′	71° 42′	1
H202	33° 01′	70° 54′	3	H295	30° 41′	71° 22′	1
H203	32° 15′	71° 32′	2	H296	30° 41′	70° 52	1
H204	33° 00′	70° 57′	3	H298	30° 21′	71° 29′	1
H206	33° 06′	71° 28′	3	H299	30° 15′	70° 41′	1
H207	31° 54′	72° 22′	2	H300	28° 55′	70° 45′	1
H208	32° 58′	71° 10′	3	H301	30° 41′	71° 22′	1
H209	33° 06′	71° 28′	3	H302	30° 41′	70° 51	1
H210	33° 39′	70° 21′	3	H303	31° 54′	70° 22′	2
H211	32° 58′	71° 10′	3	H304	31° 48′	71° 21′	2
H213	32° 25′	70° 55′	2	H305	30° 37′	71° 14′	1
H216	32° 18′	71° 31′	2	H307	29° 55′	71° 14′	1
H217	34° 04′	70° 56′	3	H308	31° 47′	70° 35′	2
H218	33° 04′	70° 57′	3	H309	30° 37′	71° 14′	1
H220	36° 45′	72° 18′	5	H310	31° 56′	71° 31′	2
H222	36° 45′	73° 09′	5	H311	30° 48′	71° 40′	1

[a]Geographic regions were established according to the environmental classification proposed by Gastó et al. (1990) based on the Köppen World Climatic Classification (1923) and the Grassland Classification System (Gallardo and Gastó 1985).

concentration of 12% (w/v, $C = 2.67$) (Laemmli 1970). Electrophoresis was performed at a constant current of 25 mA per gel at 10 °C for 25 min after the tracking dye migrated off the gel. Gels were stained overnight with 12% (w/v) trichloroacetic acid solution (5% (v/v) ethanol and 0.05% (w/v) Coomassie Brilliant Blue R-250). Destaining was carried out with tap water.

Data analysis

(1) All the lines were scored for presence or absence of the different protein components (gliadin and glutenin) identified. The data were scored as 1 (present) or 0 (absent) in a binary matrix for each line. The phenotypic dissimilarity (d_{ij}) between the binary vectors of two individuals, i and j, was computed using the Jaccard's distance index (Jaccard 1908):

$$0 \leq d_{ij} = 1 - \frac{y_{ij}}{y_{ij} + y_i + y_j} \leq 1$$

where y_{ij} represents the number of common protein subunits of individual i and j, and the number of subunits which exist only in individuals i or j, respectively, are given by y_i and y_j. Matrices of Jaccard distances between phenotypes were constructed for gliadins, glutenins and gliadins + glutenins to be used in subsequent AMOVA analyses and for the construction of distograms.

(2) Six geographical regions were identified according to the passport data of the lines and the

environmental classification proposed by Gastó et al. (1990), based on the Köppen World Climatic Classification (1923) and the Grassland Classification System (Gallardo and Gastó 1985). The analysis of molecular variance (AMOVA) was used to partition the total phenotypic variance into within-regions and among-regions (Excoffier 1992). The AMOVA analyses (WinAMOVA ver. 1.55 package (Excoffier 1992)) were performed on Jaccard distance matrices based on gliadins, glutenins and gliadins + glutenins. The variance components were statistically tested using non-parametric randomisation tests with 1000 permutations.

The AMOVA procedure provides estimates of population differentiation (ϕ_{st}) equivalent to *F*-statistics commonly used in ANOVA. Pairwise comparisons between regions examined with AMOVA resulted in values of ϕ_{st} that are equivalent to the proportion of the total phenotypic variance that is partitioned between two populations. ϕ_{st} values between each pair of populations were interpreted as the inter-population distance average between any two populations (Huff 1997; Gustine and Huff 1999).

Homogeneity of intrapopulation molecular variances (homoscedasticity) was tested using the HOMOVA procedure (modified Bartlett's test) also implemented in WinAMOVA (Stewart and Excoffier 1996). Bartlett's null distributions were obtained after 1000 permutations.

(3) Pairwise geographic distances between lines were calculated from the passport data using the Great Circle Distance formula. Pairwise geographic distances were divided into five spatial distance classes (<200, 200–400, 400–600, 600–800, >800 km). The correlations between geographic distance matrix and phenotypic distance matrices were obtained by Mantel tests (1967) using the NTSYS-pc package (Rohlf 1998).

(4) Geographic distance matrix and phenotypic distance matrices were used in construction of distograms (Degen and Scholz 1998; Vendramin et al. 1999). Average phenotypic distance of all pairs of lines in each spatial interval (d_k) was calculated as follows (Degen and Scholz 1998):

$$d_k = \frac{1}{N_k} \sum_{i=1}^{N_k - 1} \sum_{j=i+1}^{N_k} d_{ij}$$

where d_{ij} represents the Jaccard distance between lines i and j. In the case of positive spatial autocorrelation, the estimate d_k increases with increasing geographical distance between the lines. A permutation procedure was applied to test significant deviation from spatial random distribution (Manly 1997). Each permutation consisted of a random redistribution of pairwise phenotypic distances over pairwise geographic distances following the rationale presented by Vendramin et al. (1999). For each of the five spatial distance classes k, observed d_k values were compared with the distribution obtained after 2000 permutations. A 90% confidence interval for the parameters has been constructed as the interval from the 100th and 1900th ordered permutation estimates.

Results and discussion

Genetic relationships among H. chilense *accessions*

In the 66 lines considered, 91 different protein subunits were identified: 49 corresponded to LMW lutenin subunits and 42 to gliadin ones. The passport data of these lines, as well as the geographic region in which they were collected, is shown in Table 1. Most of the lines were collected from regions 1–3, between 29° and 34° latitude south; while regions 4–6 included a lower number of accessions. The phenotypic distance between *H. chilense* accessions showed a wide range: in fact, phenotypic dissimilarity (d_{ij}) ranked between 0.0000 and 1.0000 for gliadins and glutenins and from 0.0000 to 0.9714 when gliadin and glutenin data were joined. The average d_{ij} values were 0.7585, 0.8430 and 0.8007 for gliadins, glutenin and gliadin + glutenin (hereafter referred to as GG), respectively.

AMOVA analysis

Three different AMOVA analyses were performed (Table 2) because three levels of variation were considered: glutenin, gliadin and GG. In all cases, most of the genetic diversity was attributable to differences among entries within a geographic region: 94.17% for gliadins, 92.21% for GG and 89.80% for glutenins (Table 2). Nevertheless, the significant ϕ_{st} values among populations (0.058 for gliadin; 0.078 for GG; 0.102 for glutenin; $p < 0.001$ in all

Table 2. AMOVA and HOMOVA analysis for the partitioning of glutenin, gliadin and glutenin plus gliadin variation among and within *H. chilense* regions from Chile.

Data source	Source of variation	df	Variance components	% Total variance	ϕ Statistic	p value	Bartlett's index	p value
Glutenin	Among regions	5	0.039	10.20	0.102	< 0.001	0.12965	0.015
	Within regions	60	0.348	89.80				
Gliadin	Among regions	5	0.025	5.83	0.058	< 0.001	0.01066	0.651
	Within regions	60	0.402	94.17				
Glutenin plus gliadin	Among regions	5	0.032	7.79	0.078	< 0.001	0.03788	0.033
	Within regions	60	0.375	92.21				

Table 3. Inter-region distance matrix ϕ_{st} for the six *H. chilense* regions using glutenin, gliadin and glutenin plus gliadin data.

		p values					
Data source	Region	1	2	3	4	5	6
Glutenins	1		0.0000	0.0000	0.0000	0.1419	0.0000
	2	0.0969		0.0000	0.0000	0.0000	0.1229
	3	0.1533	0.0572		0.0669	0.0000	0.0000
ϕ_{st} value	4	0.1625	0.1034	0.0681		0.0000	0.1319
	5	0.0671	0.0446	0.0744	0.0944		0.2488
	6	0.1435	0.0535	0.1170	0.1187	0.0113	
Gliadins	1		0.0000	0.0000	0.0000	0.0719	0.0729
	2	0.0581		0.0000	0.0000	0.1119	0.0000
	3	0.0622	0.0522		0.0000	0.0000	0.0000
ϕ_{st} value	4	0.0687	0.0663	0.0489		0.3187	0.7353
	5	0.0503	0.0546	0.0860	0.0149		0.3656
	6	0.0522	0.0768	0.0863	0.0015	0.0031	
Glutenins plus gliadins	1		0.0000	0.0000	0.0000	0.1409	0.0000
	2	0.0749		0.0000	0.0000	0.0000	0.0000
	3	0.1039	0.0551		0.0000	0.0000	0.0000
ϕ_{st} value	4	0.1109	0.0850	0.0573		0.0000	0.4785
	5	0.0544	0.0507	0.0788	0.0571		0.1928
	6	0.0908	0.0629	0.1002	0.0633	0.0056	

Lower matrix diagonal: ϕ_{st} value proportion of the total variance that is partitioned between two regions. Upper matrix diagonal: corresponding p values.

the cases) indicates the existence of some degree of phenotypic differentiation. In addition, HOMOVA analysis for glutenin and GG shows that the molecular variances were significantly heterogeneous (Bp = 0.1297, p = 0.015; Bp = 0.0379, p = 0.033; respectively) (Table 2) among populations. Consequently, we performed the AMOVA analyses between each pair of regions for glutenin, gliadin and GG (Table 3). In this way, pairwise ϕ_{st} values were significant in 66.67, 60 and 80% for glutenins, gliadins and GG, respectively (Table 3). The maximum differentiation between regions is found for glutenins with a ϕ_{st} value of 0.1625 (between regions 1 and 4). The degree of differentiation between each pair of regions is higher for glutenin than for gliadins in most cases although the ϕ_{st} values are low in all cases. The simultaneous consideration of gliadin and glutenin data yields a higher number of significant ϕ_{st} values between regions (12 out of 15, 80%). In the other hand, the simultaneous consideration of GG is less informative than the single consideration of gliadins and glutenins separately, since the ϕ_{st} value obtained is more or less an average between the ϕ_{st} values for gliadins and glutenins. Consequently, if one of these components does not show variation among regions while the other does, it would be possible for the ϕ_{st} value for GG to be not significant. Furthermore, a significant ϕ_{st} value for GG would mean differentiation between regions. However, we are unable to know whether glutenins, gliadins or both cause it. Therefore, the consideration of glutenin, gliadins and GG

separately is the most informative situation for AMOVA analysis.

Variation of endosperm storage proteins with geographic distance

We also studied the relation between endosperm storage proteins and kilometric distances. This situation is more restrictive than the consideration of geographic regions for evaluation of the relation between variation and geographic origin. This restriction is explained by the fact that seed dispersion would be facilitated within a geographic region. The correlations between geographic distance matrix and phenotypic distance matrices for glutenins, gliadins and GG were, respectively, $r = 0.1857$; $r = 0.1287$; $r = 0.1980$ ($p < 0.001$). Likewise, correlation between phenotypic distance matrices for gliadins and glutenins was $r = 0.3470$ ($p < 0.001$). All this values are low, corresponding to a determination coefficient (R^2) of 0.034 for glutenins, 0.017 for gliadins and 0.039 for GG. Therefore, 3.9% of the variation of endosperm storage proteins (GG) is explained by kilometric distance, while this value was 7.79 in AMOVA, considering regions (Table 2). Nevertheless, this analysis also shows a significant low correlation between endosperm storage proteins variation and geographic localisation.

Construction of distograms

Distograms were constructed for glutenins, gliadins and GG (Figure 1) to inspect the phenotypic differentiation between accessions according to geographic distance. There is at least some evidence that phenotypic distances between accessions increase with geographical distance but the average phenotypic distance (d_k) of each of the spatial distance class were not lower (or higher) than what was expected by chance (Figure 1). For both type of proteins (gliadins and glutenins) a depression is observed in the distance class > 800 km, probably due to the small sample size, because only 77 out of 2145 pairwise geographical distances belong to this class.

Conclusion

Knowledge of the structure of genetic diversity within the distribution zone of *H. chilense* may be important in deciding on breeding strategies. Previous works with this species could not establish any clear relation between variability of gliadin (Atienza et al. 2000), high-molecular-weight glutenin subunits (Alvarez et al. 2001) and low-molecular-weight glutenin subunits (Atienza et al. 2002) and geographic origin. These works concluded that there was a continuous distribution of the variability for storage proteins within the entire distribution zone. Nevertheless, these works were mainly focused on the characterisation of the variability for endosperm storage protein composition and, therefore, the distribution of the variability within geographic regions was studied in a descriptive way. On the contrary, applying more powerful methods to get insight in the geographic distribution of the variability for endosperm storage proteins of this species, we have found some degree of association between endosperm storage protein and geographic localisation with several analyses. First, the AMOVA results indicate that the percentage of variation for gliadins, glutenins and GG explained by regions was 5.8, 10.2 and 7.8, respectively (Table 2). In the same way, the direct correlation between the geographic distance matrix and the phenotypic distance one also yielded significant values ($r = 0.1857$; $r = 0.1287$; $r = 0.1980$; $p < 0.001$). Distograms also showed some degree of differentiation among accessions according to geographic distance. From these results, it may be suggested that a low part of the variation is related to geographic distance, while the majority is not.

This was expected, since endosperm storage proteins are neutral towards the environment and therefore, they do not provide any selection advantage. These results show a high degree of polymorphism for endosperm storage proteins. Consequently, these proteins are very useful as molecular markers for the study of intra-specific variability in *H. chilense*. In spite of the neutral behaviour of these proteins towards the environment, we have detected a low degree of differentiation among regions, which could not be detected using the descriptive analyses in previous works (Atienza et al. 2000, 2002; Alvarez et al. 2001). This variation may be caused by the probable association between endosperm storage protein and genes for adaptation. Therefore, it may be suggested that collection of new entries from as many different regions as possible would be an

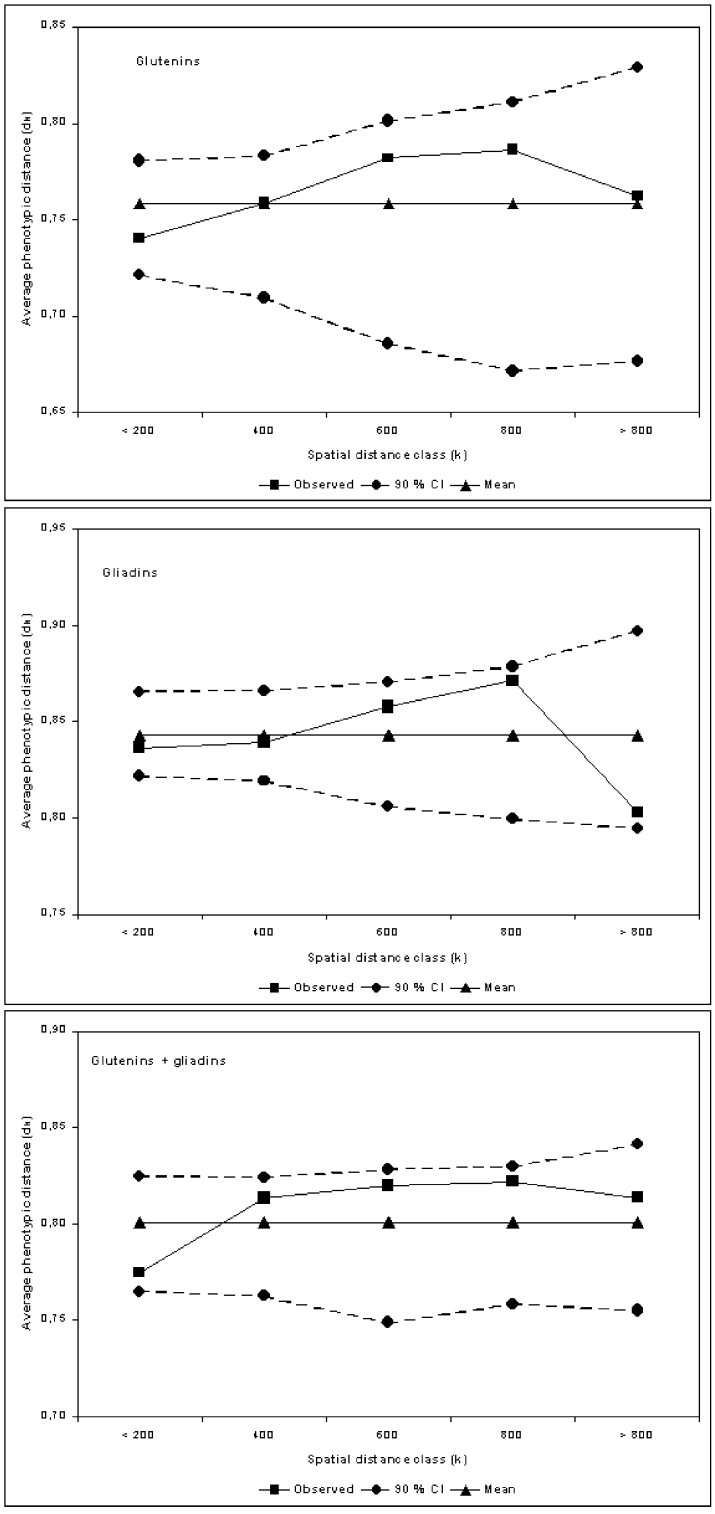

Figure 1. Distograms of phenotypic distances (d_k) in five geo-graphical distance classes and their 90% confidence interval (CI).

adequate strategy to increase the variability for endosperm storage proteins of this species for breeding programmes of wheat and tritordeum.

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