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Diversification of seed carotenoid content and profile in wild barley (*Hordeum chilense* Roem. et Schultz.) and *Hordeum vulgare* L.-*H. chilense* synteny as revealed by DArTSeq markers

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Abstract

The high carotenoid content and distinctive carotenoid profile of tritordeum are conferred by its wild progenitor, *Hordeum chilense*. Genetic studies on this wild barley could exploit the knowledge gained in *Hordeum vulgare* L. if the synteny between *H. vulgare* and *H. chilense* is established. DArTSeq markers were aligned to barley genome and used to inspect *H. chilense*-barley synteny. All chromosome pairs showed a good degree of collinearity with the exception of 7Hv-7Hch, where a reciprocal translocation in 7Hch was identified.

Carotenoid analyses revealed a high diversity for total carotenoids, free and esterified lutein in a collection of *H. chilense*. Population structure analyses revealed the existence of two subgroups contrasting for total carotenoids, free lutein and esterified lutein in seeds. Lutein esters were produced with palmitic and linoleic acids as happens in tritordeum. However, tritordeum prefer palmitic acid for the synthesis of lutein esters but this preference is not maintained in *H. chilense*. This indicates the existence of diversity in the enzymes involved in the esterification which could be useful in tritordeum breeding. Furthermore, several accessions produced lutein monoesters but they lacked diesters which suggests that esterification is controlled by more than one enzyme in *H. chilense*. A total of 91 marker-trait associations were identified for carotenoid content and profile. These associations constitute a good starting point for future genetic analyses for the identification of candidate genes from *H. vulgare* genome.

Keywords: Carotenoid esters, lutein esters, *Hordeum chilense*, tritordeum, wild relatives

1 **Introduction**

2 The progressive narrowing of the genetic diversity in crops (Tanksley and McCouch 1997;
3 Warburton et al. 2006) is a serious threat to agriculture. Modern varieties present a
4 combination of favourable alleles adapted to intensive cropping systems but their potential to
5 evolve in a changing environment has decreased as the result of reduced genetic diversity
6 (Charmet 2011). Breeders are reluctant to go back to wild relatives for new variation
7 (Rasheed et al. 2017) but wild species constitute an important source of diversity for breeding
8 (McCouch et al. 2013). Wild relatives can be crossed with wheat to increase the diversity
9 available for breeding (King et al. 2017; Martín et al. 2018; Rodríguez-Suárez et al. 2011).
10 The utilization of more distantly related species is also possible, such as the development of
11 translocation lines of *Agropyron cristatum* L. Gaertn. (Ochoa et al. 2015) or *Hordeum*
12 *chilense* Roem. et Schultz. into common wheat (Mattera et al. 2015a; Mattera and Cabrera
13 2017; Rey et al. 2015; Said et al. 2012). Intergeneric hybridization has allowed the
14 development of new crops such as triticale and tritordeum [*xTritordeum martini* A. Pujadas
15 (Pujadas 2016)], the latter being the amphiploid derived from the hybrid between *H. chilense*
16 and durum wheat (Martin and Sánchez-Monge 1982). This new crop is being commercialized
17 at present by Agrasys S.L. (Vivagram ®) due to the golden color of its flour and other
18 functional properties (www.tritordeum.com). The differential properties of tritordeum are
19 conferred by *H. chilense* (Martin et al. 1996). This is particularly important for the high
20 carotenoid content of tritordeum seeds (Atienza et al. 2007b) since it confers the golden
21 colour which is one of the main commercial attributes of tritordeum derived products.

22 *H. chilense* is a native South American diploid wild barley that is distributed from 29° to 43°
23 Latitude South in Chile and Argentina. This species shows a great diversity for agro-
24 morphological and endosperm storage proteins (Atienza et al. 2000; Atienza et al. 2002; Vaz
25 Patto et al. 2001). *H. chilense* is responsible for both the high carotenoid content of the seeds

of tritordeum (Atienza et al. 2007b; Rodríguez-Suárez et al. 2014) and for the distinctive esterification profile of carotenoids (Mattera et al. 2015b; Mellado-Ortega and Hornero-Méndez 2015). Carotenoid esters increase carotenoid retention during the storage of grain (Mellado-Ortega et al. 2015) and flour (Mellado-Ortega and Hornero-Mendez 2017) so that it is an important trait for tritordeum breeding. Collectively, our results show that tritordeums have a larger amount of carotenoids than durum wheat, a high proportion of carotenoids esterified with fatty acids and a preference towards palmitic acid for the synthesis of lutein esters (Mattera et al. 2015b; Mattera et al. 2017; Mellado-Ortega and Hornero-Mendez 2012). However, carotenoid content and profile remain unexplored in *H. chilense* despite previous studies for other traits suggesting that a high degree of diversity may exist for these traits.

Candidate gene approaches have permitted the identification and mapping of the main genes producing the high lutein content in tritordeum (Atienza et al. 2007a; Rodríguez-Suárez and Atienza 2012; Rodríguez-Suárez et al. 2014). These advances have been possible due to the carotenoid pathway being well known (Cuttriss et al. 2011; Hirschberg 2001). However, the candidate gene approach is not viable when the pathways underlying the traits of interest are not fully understood. In this context, synteny-based candidate gene identification would be an interesting option for *H. chilense* provided that macro-collinearity relationships between *H. chilense* and *H. vulgare* L. are established. This approach has been proven to be useful for identifying collinear regions between orphan crops or wild relatives and model species as a first step towards the identification of candidate genes responsible for traits of interest (Muchero et al. 2009; Webb et al. 2016). The existence of synteny among species belonging in the Triticeae (Devos 2005) and the recent release of the barley reference genome assembly and gene predictions (Mascher et al. 2017) (<http://pgsb.helmholtz-muenchen.de/plant/barley/download/index.jsp>) provide new opportunities for genetic studies in *H. chilense*.

Several collecting missions covering the complete distribution area of *H. chilense* (Giménez et al. 1997; Tobes et al. 1995) allowed the diversity available for tritordeum breeding to be increased (Martin et al. 1998). Diversity studies in *H. chilense* have described the existence of different ecotypes but they do not agree on the number of groups (Castillo et al. 2010; Vaz Patto et al. 2001). Indeed, while AFLP and morphological data suggested the existence of three ecotypes in *H. chilense* (Vaz Patto et al. 2001), later studies using microsatellite markers concluded that *H. chilense* diversity was structured in two different groups (Castillo et al. 2010). Genotyping the diversity panel in *H. chilense* with molecular markers covering the complete genome of the species would allow one to distinguish between both alternatives. Besides, this information would be very useful for exploiting natural variation in this species for carotenoid content and profile. The goals of the current study were to investigate: (i) the synteny relationships between *H. chilense* and barley using whole-genome coverage DArTSeq markers; (ii) the genetic structure of a natural collection of *H. chilense* accessions representative of its distribution area; (iii) the carotenoid content and profile of this collection; and (iv) the identification of marker-trait associations for carotenoid content and profile.

1 **Materials and methods**

2 Plant material and field testing

3 The plant material included a diversity panel and a mapping population. The diversity panel
4 consisted of 93 accessions of *H. chilense* from its complete distribution area. The provenance
5 of these accessions has been previously reported (Vaz Patto et al. 2001). The mapping
6 population is a recombinant inbred (RI) comprising 92 individuals (Rodríguez-Suárez et al.
7 2012).

8 Plants were sown in pots with a mixture 1:4.5 (v:v) (sand:premium pflanzerde substrate).
9 Osmocote Exact Mini (1.2 g/L) (Everris International B.V.) was added. Plants were
10 maintained in growth chamber (light period: 22°C during 11 h + 16°C during 1 hour; dark
11 period: 10°C during 11h + 16°C during 1h) until samples were taken for DNA isolation. For
12 the field trials, the diversity panel was initially sown as described above and then transplanted
13 into outdoors facilities, following a completely randomized block design with two
14 replications. Plants were maintained under anti-bird net structure. Grain samples were
15 harvested at maturity. Since *H. chilense* is a wild barley, its spikes disarticulate at maturity.
16 For this reason, grains were harvested by hand daily for each accession as they reached
17 maturity.

18

19 DNA isolation and genotyping

20 Genomic DNA was isolated from bulked leaf tissue of seedlings following the CTAB
21 protocol with slight modifications (Murray and Thompson 1980) using TissueLyser II mill
22 (Qiagen), two stainless-steel balls (5 mm diameter) for sample disruption and 2 mL

Eppendorf tubes. Genotyping by sequencing analysis of the Diversity Panel and the Mapping Populations was performed at Diversity Arrays Technology Pty Ltd (Canberra, Australia).

Establishment of macro-syntenic relationships between *H. chilense* and *H. vulgare*

To inspect macro-syntenic relationships between *H. chilense* and barley, we compared the relative position of DArTSeq markers in the genetic map of *H. chilense*, with the location of the corresponding sequence in the barley genome after alignment analyses.

The genetic map was constructed using Joinmap 4.0 (Van Ooijen 2006) and MSTmap (Wu et al. 2008). Only DArTSeq markers consistently segregating in the parental lines of the mapping population and with a call rate of above 90% were used for mapping along with markers previously mapped (Rodríguez-Suárez and Atienza 2012; Rodríguez-Suárez et al. 2012). JoinMap 4.0 mapping software (Van Ooijen 2006) was used for segregation and linkage analysis. Mendelian segregation was tested by Chi-square goodness-of-fit to a 1:1 ratio. Markers significantly deviating from mendelian segregation were excluded. An LOD threshold of above 9.0 was used to separate the markers into seven distinct chromosomes. Marker order within each chromosome was determined with MSTmap (Linux version) (Wu et al. 2008) with the following parameters: population type (RIL8); distance function kosambi; no_map_distance 35.0; no_map_size 2.0; missing_threshold 10.0; estimation_before_clustering no; detect_bad_data yes; objective_function COUNT.

H. chilense-derived DArTSeq markers successfully mapped in the *H. chilense* map were aligned to barley genome. A BlastN search (Altschul et al. 1990) was performed using BLAST+ (Camacho et al. 2009). DArTSeq sequences were used as a query against the barley coding sequences (nucleotides) of annotated high and low confidence genes obtained from (http://webblast.ipk-gatersleben.de/barley_ibsc/downloads/) (Mascher et al. 2017).

Relationships between *H. chilense* and barley were inspected using CIRCOS (Krzywinski et al. 2009). Only the best hit to the barley genome was used to inspect the relationships between *H. chilense* and barley homoeologues chromosomes. In addition to this, Spearman rank correlations were calculated between the relative order of markers in each chromosome of the *H. chilense* map and the corresponding order of the best matches in the barley genome using Statistix v 10.0.

Genetic structure of the diversity panel

A model-based clustering, employing a Bayesian algorithm, was applied to infer the genetic structure in the diversity panel of *H. chilense* accessions using Structure software version 2.3.4 (Pritchard et al. 2000). A set of 474 DArTSeq markers distributed throughout the seven chromosomes was used. The program was run assuming a population admixture model. The number of assumed groups (K) was set to vary between 1 and 4, and for each value of K twenty independently MCMC (Markov Chain Monte Carlo) of 500,000 iterations were run, of which the first 50,000 were discarded as burn-in. The likelihood of the data for a given number of assumed groups (K) is provided by the software, and the value of K with the highest likelihood can be interpreted as an estimation of the underlying number of groups. A genotype was considered to belong to a group when its membership coefficient was ≥ 0.5 . The membership probabilities of each genotype to each group inferred were obtained and used as covariate for the preliminary identification of marker-trait associations, as described below. In addition to this, a principal component analysis (PCoA) was obtained based on genotype data from the DArTSeq markers using Tassel 5.2.40 (Bradbury et al. 2007) as an alternative method to inspect the existence of structure in *H. chilense*. Markers used for Genome Wide Association Scan (GWAS) analysis (described below) were used for PCoA.

1

2 Extraction of carotenoids

3 Carotenoid pigments were extracted from *H. chilense* grains (only the diversity panel) using
4 the methods described by (Mellado-Ortega and Hornero-Méndez 2015). Briefly, the plant
5 material (0.15 g; ca. 50 seeds) was ground with a ball mill (MM400 Retsch) by placing the
6 seeds in a 2 mL safe-lock Eppendorf® tube together with two stainless-steel balls (5 mm
7 diameter) during 1 min at 25 Hz rate. Carotenoids were subsequently extracted with 1 mL of
8 acetone (containing 0.1% BHT), centrifuged at 13,500 g for 5 min at 4°C, and the supernatant
9 was directly used for the chromatographic analysis. Only a one-step solvent treatment was
10 necessary for the complete extraction of pigments. All operations were carried out under
11 dimmed light to prevent isomerization and photodegradation of carotenoids. Analyses were
12 performed in duplicate.

13 Pigment identification

14 The identification of carotenoid pigments and their esters in *H. chilense* grains has already
15 been presented by Mellado-Ortega and Hornero-Méndez (2015). Briefly, the procedure
16 consisted of: (i) separation of pigment by thin-layer chromatography (TLC) and
17 cochromatography with purified pigments; (ii) observation of the pigment color on TLC
18 plates under white, UV254 nm, and UV360 nm wavelength; (iii) obtainment of UV-vis
19 spectra in different solvents and comparison with the values reported in the literature; (iv) and
20 chemical derivatization microscale test for the examination of 5,6-epoxide, hydroxyl, and
21 carbonyl groups (Britton 1995; Davies and Köst 1988; Eugster 1995; Foppen 1971). Carbonyl
22 and hydroxyl groups were also investigated by Fourier transform infrared spectroscopy.
23 Zeaxanthin and lutein standards were obtained in the laboratory by means of TLC from a de-
24 esterified carotenoid extract from red pepper (*Capsicum annuum* L.) and mint (*Mentha*

arvensis L.) respectively (Mínguez-Mosquera et al. 1992; Mínguez-Mosquera and Hornero-Méndez 1993). Carotenoid esters were identified by liquid chromatography-mass spectrometry (LC-MS (APCI+)) as described by (Mellado-Ortega and Hornero-Méndez 2015).

Analysis of carotenoids

Carotenoid analysis was carried out by HPLC according to the method of Mellado-Ortega and Hornero-Méndez (2015). The HPLC system consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector, and controlled with Empower2 software (Waters Cromatografia, S.A., Barcelona, Spain). A C18 reversed-phase analytical column (Mediterranea SEA18, 3 μ m, 20 x 0.46 cm; Teknokroma, Barcelona, Spain) was used. Pigment separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and 25% deionized water, which was increased linearly to 95% acetone in 10 min, then raised to 100% in 2 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. An injection volume of 10 μ L and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired in the 350-700 nm wavelength range. Quantification was done by using calibration curves (peak area at 450 nm versus the pigment concentration; range of 0.5-45 μ g/mL) prepared with lutein, β -carotene and zeaxanthin standards isolated and purified from natural sources (Mínguez-Mosquera and Hornero-Méndez 1993). The concentrations of lutein esters and cis-isomers of lutein were estimated by using the calibration curve for free lutein, since the esterification of xanthophylls with fatty acids does not modify the chromophore properties. Analyses were made in duplicate and data were expressed as μ g/g dry weight. Statistical analyses of variance for total carotenoid content and concentration of each compound were conducted using Statistix version 10.0 (Analytical Software, Tallahassee, FL, USA).

Differences between genotypes or geographical origin were established using Tukey's honest significance (HSD) test at $P < 0.05$. Dunnett's Multiple Comparison test was used to identify genotypes with linoleate: palmitate ratio ($[\text{Lutein-linoleate} + \text{Lutein--dilinoleate}] / [\text{Lutein-palmitate} + \text{Lutein-dipalmitate}]$) significantly different to 1:1 (hypothetic control).

Identification of marker trait associations (MTA) for carotenoid content and profile in *H. chilense* using GWAS

Marker-trait associations were determined using TASSEL 5.2.45 (Bradbury et al. 2007). Only SNP markers successfully mapped were considered for association tests. Markers with more than 10% missing data points and markers with a minimum allele frequency of less than 5% in the diversity panel were excluded prior to association analyses, which resulted in a set of 12,638 markers. Association analyses were performed using the MLM model including the Q-matrix derived from the Structure analysis described above and the kinship matrix calculated with Tassel (MLM + Q + K). Marker-trait associations were declared significant at the threshold determined by the Bonferroni correction.

Results

Macro-synteny relationships between *H. chilense* and barley

Both the diversity panel and the mapping population were genotyped with DArTseq® platform. An initial set of 103,429 silico DArT markers and 35,205 SNPs was generated. Out of these, 22,213 silico DArT and 6,443 SNPs segregated between the parental lines of the mapping population (H1 and H7 *H. chilense* accessions), and were consistently detected in three replicates of each accession. The marker dataset was filtered using a 7.5% cutoff of missing data in the mapping population which resulted in further reducing the dataset (18,035 silico DArT and 5,381 SNPs). A total of 16,631 markers were successfully mapped in the *H. chilense* map. DArTSeq markers were aligned to the barley genome using Blastn as implemented in BLAST+. A total of 1,425 DArTSeq markers produced a significant match with barley genes (see Supplementary file 1) and were used to inspect synteny relationships.

Macro-synteny relationships between *H. chilense* and barley are shown in Fig. 1. To investigate the degree of collinearity between both species, Spearman's-rank correlations were calculated between the relative order of markers in the *H. chilense* map and the relative position of the barley sequences in the barley genome. In general, a good degree of collinearity was found between both species with values of above 0.8 for all chromosome pairs with the exception of 7H^{ch}-7Hv, in which no correlation was found (Table 1). Thus, this chromosome was inspected in detail. Fig. 2 shows three differentiated blocks within chromosome 7Hch. The central block shows good collinearity with 7Hv. However, the distal parts of 7HchS (black segment) and 7HchL (pink segments) show correspondence with 7HvL and 7HvS respectively (Fig. 2). This suggests the existence of a reciprocal translocation between 7HchS and 7HchL in *H. chilense*. To further test this hypothesis, we generated a hypothetical original chromosome 7Hch by reverting the hypothetical translocation and re-

calculated Spearman-rank correlations with the position of homoeologue genes in barley. This resulted in a correlation of 0.86 between 7Hch and 7Hv (Table 1), which suggests that there is a good degree of collinearity within the three blocks described above and barley.

Analysis of population structure

The underlying population structure was firstly studied with the Bayesian approach implemented in the Structure software (Pritchard et al. 2000). Evidence of two subpopulations was provided by this analysis since the ΔK value occurred at $K = 2$. Accessions H1 and H7 were considered as being representatives of each of these groups since they had a membership coefficient of 1.0 to each group. Each genotype was assigned to either groupH1 or groupH7 based on membership probability >0.5 (Fig. 3). GroupH7 includes 50 accessions, while group H1 consists of the remaining 43 accessions. Most of the latter were assigned to each subpopulation with a membership probability coefficient of above 0.9. However, accessions H31, H46 and H221 showed an intermediate behaviour (with membership estimates around 0.5) and 9 accessions assigned to groupH7 had membership probabilities of below 0.9 (Fig. 3).

PCoA analysis was carried out as an alternative way of inspecting the existence of a population structure (Fig. 3). The first two principal coordinates explained 50.12% of total variation, PC1 being more important (44.67 % of total variation) than PC2 (5.60% of total variation). Accessions in the PCoA graph were depicted in red or blue according to their assignment to groupH7 or groupH1 in the structure analysis, respectively. The three accessions with membership coefficients of around 0.5 (H31, H46 and H221) were clearly separated from the rest in the PCoA graph (Fig. 3). Similarly, the accessions represented as

triangles in the PCoA graph belong to groupH7 but with membership coefficients of below 0.9.

The passport data of the accessions collected during several expeditions to Chile were used to compare the relationship between geographical distribution and the population structure analysis (Fig. 4). GroupH7 covered a larger distribution than groupH1. Accessions in groupH1 were mostly located in the central part of the distribution area while accessions grouped with H7 were mainly found above and below the distribution area of groupH1.

Variation for carotenoid content and profile in *H. chilense*

The carotenoid composition, including carotenoid esters, was analysed in all the accessions of the diversity panel. Fig. 5 shows the chromatograms corresponding to seeds of H1, H7 and H290. Both H1 and H7 are considered as representatives of the two biotypes found after structure analysis. Both accessions showed a similar profile with free lutein as the main carotenoid and the presence of lutein monoesters and diesters. The basic statistical parameters of the quantitative carotenoid traits are shown in Table 2. Total carotenoid content ranged between 5.07 and 43.68 µg/g. Lutein diesters were not detected in five accessions (H55, H211, H290, H293 and H310). In addition, the accession H290 was also characterized by the absence of lutein monoesters.

GroupH1 and groupH7 were compared for carotenoid profile. GroupH7 was characterized by higher contents of total carotenoids, free lutein and esterified lutein (Fig. 6). However, these groups did not differ for the relative contribution of free and esterified lutein relative to the total amount of lutein (Fig. 6).

Previous studies in *tritordeum* have shown a skewed production of lutein esters towards palmitate which suggested that *H. chilense* preferentially formed lutein esters containing palmitic acid. Thus, the ratio linoleate:palmitate ($[\text{Lutein-linoleate} + \text{Lutein-dilinoleate}] / [\text{Lutein-palmitate} + \text{Lutein-dipalmitate}]$) was calculated to investigate whether this assumption was correct or it was genotype-dependent. Accession H35 exhibited a balanced synthesis of lutein esters with either palmitic or linoleic acids. Out of 92 accessions producing lutein esters, 43 did not differ from H35, which indicates a lack of preference for the fatty acid involved in esterification. On the contrary, 46 lines showed a preference towards palmitic acid and only three accessions, H210, H217 and H218, favoured linoleic acid for carotenoid esterification (Fig. 7).

Association analysis for carotenoid profile

Genome-wide association analysis was performed for lutein profile (free lutein, lutein palmitate, lutein linoleate, lutein dilinoleate, lutein dipalmitate and lutein linoleate-palmitate). A total of 12,638 DArTSeq markers mapped in the *H. chilense* map were retained for the GWAS analysis after removing SNPs with a minor allele frequency lower than 0.05 and SNPs with more than 10% of missing data. Marker-trait associations were determined by MLM taking into account the population structure matrix (Q matrix obtained from the structure analysis) and the kinship matrix (K matrix calculated with TASSEL). No significant MTA associations were found for lutein palmitate and lutein dilinoleate. Manhattan plots for the remaining traits are shown in Fig. 8. A total of 91 significant MTA were detected (see Supplementary file 2). Most MTA were detected for free lutein (79 MTA) while the number of significant MTA was much lower for lutein monolinoleate (1 MTA), lutein dipalmitate (8 MTA) and lutein linoleate-palmitate (3 MTA).

Regarding free lutein, significant MTA were detected in all chromosomes but 7Hch. The highest number of MTAs were located on chromosomes 1Hch and 6Hch. Out of 12 MTA for lutein esterification traits, two markers on 3Hch (14439596 and 14448303) were significantly associated with the variations in lutein linoleate and lutein linoleate-palmitate (Supplementary file 2).

Discussion

Syntenly between *H. chilense* and *H. vulgare*

Comparative studies have allowed the establishment of the relationships between the genomes of related species such as grasses (Devos 2005; Moore et al. 1995) or legumes (Choi et al. 2004). Our results indicate that *H. chilense* shows a good degree of macro-collinearity with barley with the exception of chromosome 7H^{ch}, in which a significant reorganization was detected. This reorganization was previously hypothesized since the gene *Phytoene synthase 1* (*Psy1*) was located in the short arm of chromosome 7H^{ch} (Atienza et al. 2007a; Mattera et al. 2015b), while the homoeologues genes in wheat are located in the long arm of 7A, 7B and 7D (Pozniak et al. 2007; Rodríguez-Suárez et al. 2010). However, our previous studies have been conducted using *H. chilense*-wheat genetic stocks, including chromosome addition, translocation or substitution lines (Atienza et al. 2007a; Mattera et al. 2015b). Consequently, we were not able to prove that the reorganization of 7H^{ch} was already present in the *H. chilense* genome since it might have occurred during the development of the different genetic stocks. Our current results confirm that the translocation in 7H^{ch} is present in the H^{ch} genome and it is not the result of an interaction with the wheat genome.

The good degree of macro-syntenly between *H. chilense* and barley will allow the exploitation of the genome resources available in barley (Mascher et al. 2017; Mayer et al. 2011;

The_International_Barley_Genome_Sequencing_Consortium 2012). Specifically, it will enable the identification of candidate genes for traits of interest in barley. Similar strategies are being applied in other crops such as white lupin with the aim of synteny-based gene cloning (Książkiewicz et al. 2017) or faba bean for the identification of candidate genes for stomatal traits (Khazaei et al. 2014) .

H. chilense population structure and carotenoid profile diversity

H. chilense has a wide variation in morphological traits (Bothmer et al. 1980; Vaz Patto et al. 2001). *H. chilense* was considered as being a polymorphic taxon with a wide diversity in morphological characters (Bothmer et al. 1980). Later works studied the existence of population structure in *H. chilense* using morphological and AFLP markers (Vaz Patto et al. 2001), or microsatellite markers (Castillo et al. 2010) with slightly different results (two or three groups). Our current results show the existence of two clearly differentiated groups represented by accessions H1 and H7 in agreement with previous findings (Castillo et al. 2010). On the contrary, three ecotypes (I, II and III) were described in another work (Vaz Patto et al. 2001). Groups I and III correspond to groupH1 and groupH7, respectively. However, the accessions included in group II did not constitute a separate group in our analysis. On the contrary, these genotypes were distributed between group H1 (five accessions) and group H7 (9 accessions) (Supplementary file 3). The existence of three groups in *H. chilense* has been hypothesized using a set of fourteen repetitive probes and FISH analyses (Rey et al. 2018). However, that work does not provide enough evidence to confirm the existence of groups since only three accessions were studied. Indeed, the authors acknowledged that more individuals from each group should be analyzed to confirm the existence of different hybridization patterns in each group.

1

2 Compared to tritordeum, *H. chilense* shows a higher degree of diversity for carotenoid content
3 and profile which is essential for genetic studies. Indeed, the accessions analyzed in this work
4 showed a coefficient of variation of 49.1% while it was 26.7% in tritordeum (Atienza et al.
5 2007b). Diversity is also higher than that found in common, durum or einkorn wheat (Ziegler
6 et al. 2015). Regarding the carotenoid profile, five accessions did not produce lutein diesters
7 in contrast with all previous reports in tritordeum. Furthermore, accession H290 was also
8 characterized by the absence of lutein monoesters. The existence of accessions with the ability
9 to produce monoesters but no diesters suggests the implication of more than one enzyme for
10 lutein esterification in *H. chilense*. However, the limited number of accessions without lutein
11 esters is a handicap for the identification of the genetic basis using GWAS, and the
12 development of alternative strategies could be considered in future works.

13 Previous results showed palmitic acid selectivity in the synthesis of lutein esters in tritordeum
14 (Mattera et al. 2015b; Mattera et al. 2017; Mellado-Ortega and Hornero-Mendez 2012) which
15 suggested a preference towards palmitic acid for lutein esterification in *H. chilense*. The
16 existence of substrate specificity is not related to the availability of fatty acids in tritordeum
17 (Mellado-Ortega and Hornero-Méndez 2018). Indeed, linoleic acid content was two-fold
18 higher in the endosperm compared to palmitic acid (Mellado-Ortega and Hornero-Méndez
19 2018), but tritordeum synthesized a larger amount of lutein esters with palmitic ones,
20 although this preference towards palmitic acid for lutein esterification is not observed in *H.*
21 *chilense*. Although most accessions favored the synthesis of lutein esters with palmitic acid
22 (46 accessions), forty-three accessions showed a balanced contribution of palmitic and
23 linoleic acid to the production of esters and three accessions favored the utilization of linoleic
24 acid. This suggests the existence of diversity in the xanthophyll acyl transferases involved in

1 the esterification in *H. chilense*, which could be used to modify the esterification profile in
2 tritordeum.

3 Significant differences were detected between groupH1 and groupH7 for the carotenoid
4 profile. Indeed groupH7 doubled the production of total carotenoids, free lutein and esterified
5 lutein compared to groupH1 (Fig. 6). However, the relative contribution of free and esterified
6 lutein to the total pool of lutein was not significantly different between both groups. This
7 means that the relative proportion of lutein esters is not determined by the total amount of
8 lutein as happened in wheat-*H. chilense* chromosome substitution lines (Mattera et al. 2015b).

9 As explained above, groupH1 and groupH7 correspond to groups I and III that were
10 determined by using AFLP markers and morphological traits (Vaz Patto et al. 2001). Thus, it
11 is possible to compare groupH7 and groupH1 for morphological traits. GroupH7 is
12 characterized by broader, shorter spikes, a greater density of stomata in the abaxial leaf size
13 and a high level of avoidance of rust fungi (Vaz Patto et al. 2001). However, these
14 morphological traits do not seem to be directly related to the carotenoid content in seeds. In
15 contrast, other traits such as seed color, anthesis date or maturity date which could be
16 associated with variations in the carotenoid profile did not show differences between either
17 group.

18 The passport data of the accessions obtained from the collection missions in Chile were also
19 considered. The two groups found in *H. chilense* differed in their geographic location (Fig. 4).
20 The majority of accessions of groupH1 were collected at around 34° latitude South while
21 accessions from H7 were obtained at around 30° latitude south. According to the new Koppen
22 classification (Peel et al. 2007) the area around 30° corresponds to a temperate environment
23 with hot summers (temperature of the hottest month above 22°C) while those included in
24 groupH1 were collected from a temperate environment with warm summers (temperature of

the hottest month below 22 °C and four months in which the temperature is above 10°C). Besides, these areas are also different in terms of productivity according to (van Leeuwen et al. 2013). Seed carotenoids are important antioxidants that contribute to the antioxidant system and reduce seed ageing (Howitt and Pogson 2006). Thus, seed carotenoids contribute to successful germination (Howitt and Pogson 2006). The diversification for carotenoid content between groupH1 and groupH7 suggests differences in the antioxidant system in seeds between these groups which might be important during germination.

Marker-trait associations

Significant MTAs were identified in all chromosomes but 7Hch for free lutein content. In *H. chilense*, genes related to either carotenoid synthesis or carotenoid degradation, have been mapped on all chromosomes with the exception of 1H^{ch} (Rodríguez-Suárez and Atienza 2012; Rodríguez-Suárez and Atienza 2014; Rodríguez-Suárez et al. 2012). Significant MTAs have been detected on all of the chromosomes of durum wheat by association and linkage mapping (reviewed by (Ficco et al. 2014)). Thus, it is not surprising that the same may happen in *H. chilense*.

Two markers were associated with more than one esterification-related trait but not with free lutein content. Thus, they could be important for explaining differences in carotenoid esterification. None of these sequences showed a significant similarity with barley genes, or with other grasses (data not shown). The MTAs reported in this work have not been validated and thus we consider these results to be preliminary ones.

Conclusions

1 *H. chilense* shows a good degree of macrocollinearity with barley in all chromosomes but
2 7Hch where a reciprocal translocation was identified. Esterification is controlled by more than
3 one enzyme as deduced by the ability of some accessions to produce monoesters but no
4 diesters. Besides, there is diversity in the xanthophyll acyl transferases involved in
5 esterification. Indeed, some accessions favoured the synthesis of lutein esters with palmitic
6 acid, as happens in tritordeum, but other accessions did not show this preference. The
7 existence of marked differences for carotenoid content may imply differences in the
8 antioxidant system between the subgroups of *H.chilense*. The marker trait associations
9 constitute a good starting point for the identification of candidate genes from *H. vulgare*
10 genome for further genetic studies.

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Table 1 Spearman's Rank correlation coefficients between *H. chilense* and *H. vulgare*.

Chr.pair	1H ^{ch} - 1Hv	2H ^{ch} - 2Hv	3H ^{ch} - 3Hv	4H ^{ch} - 4Hv	5H ^{ch} - 5Hv	6H ^{ch} - 6Hv	7H ^{ch} - 7Hv	7HchR ¹ - 7Hv
r	0.821*	0.943*	0.932*	0.933*	0.942*	0.892*	0.260	0.860

*Significant correlation at $p < 0.05$.

¹7HchR refers to a hypothetical original chromosome 7Hch by reverting the reciprocal translocation shown in Figure 2.

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Table 2 Basic statistics for carotenoid in the diversity collection (ug g⁻¹ dry weight)

Trait ¹	Min	Max	St. dev	Mean
all- <i>trans</i> -zeaxanthin	0.52	4.59	0.67	1.44
all- <i>trans</i> -β-carotene	0.00	0.63	0.07	0.05
Free lutein	1.83	36.73	6.18	8.68
Lutein linoleate	0.00	4.48	0.92	1.62
Lutein palmitate	0.00	5.83	1.33	2.10
Lutein monoesters	0.00	9.59	2.18	3.71
Lutein dilinoleate	0.00	2.87	0.45	0.41
Lutein linoleate-palmitate	0.00	4.71	1.00	1.05
Lutein dipalmitate	0.00	3.63	0.74	0.76
Lutein diesters	0.00	9.57	2.13	2.23
Esterified lutein	0.00	16.96	3.91	5.94
Total lutein	4.37	39.02	7.61	14.62
Total carotenoid	5.07	43.68	8.01	16.11

Lutein monoesters= Lutein linoleate + Lutein palmitate; Lutein diesters = Lutein dilinoleate + Lutein linoleate-palmitate + lutein dipalmitate; Esterified lutein = Lutein monoesters + Lutein diesters; Total lutein = Free lutein + Esterified lutein; Total carotenoids= all-*trans*-zeaxanthin + all-*trans*-β-carotene+total lutein

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Caption Figures.

Fig. 1. Macro-syteny relationships between *H. chilense* and barley. The relative position of DArTSeq markers in the *H. chilense* map was compared with the location of the barley sequence with the best match after alignment analysis in the *H. vulgare* genome. Relationships between *H. chilense* and *H. vulgare* were displayed with CIRCOS software.

Fig. 2. Structural rearrangements in chromosome 7Hch of *H. chilense* compared to barley 7Hv. A. Links between 7HchS and 7HvL (depicted in black) and 7HchL and 7HvS (depicted in pink) suggest the existence of a reciprocal translocation between 7HchS and 7HchL. B. Hypothetical original 7Hch chromosome constructed by interchanging the position of 7HchS and 7HchL segments.

Fig. 3. Analysis of population structure in *H. chilense*. (a) Membership of *H. chilense* accessions to groupH1 (blue) and groupH7 (red) after Structure analysis. (b) Principal Component Analysis (PCoA).

Fig. 4. Distribution of *H. chilense* accessions grouped in groupH1 (left) and groupH7 (right) after analysis of population structure. Accessions were placed on map of Chile using MapTool by Darrin Ward (<https://www.darrinward.com/lat-long/>).

Fig. 5. HPLC chromatograms obtained for *H. chilense* accessions H1, H7 and H290. Peak identities: 1, (all-E)-Zeaxanthin; 2, (all-E)-Lutein; 3, (9Z)-Lutein and (13Z)-Lutein; 4, Lutein linoleate; 5, Lutein palmitate; 6, Lutein dilinoleate; 7, Lutein linoleate-palmitate; 8, Lutein dipalmitate.

Fig. 6. Comparative analysis of *H. chilense* groups for carotenoid profile: total carotenoid content (a), free lutein (b), esterified lutein (lutein monopalmitate+lutein monolinoleate) (c); ratio lutein diesters (lutein dilinoleate + lutein dipalmitate + lutein linoleate-palmitate) / total lutein (free lutein + esterified lutein) (d); ratio lutein monoester (lutein linoleate + lutein palmitate)/total lutein (e); ratio free lutein/total lutein (f). Graphs a, b and c are shown as $\mu\text{g/g}$ dry weight. Bars with the same letter are not significantly different at $P=0.05$, determined by Tukey's HSD test.

Fig. 7. Relative production of lutein esters with linoleic or palmitic acids in the diversity panel.

The relative production of lutein esters with linoleic or palmitic acids was investigated by calculating the ratio (lutein monolinoleate + lutein dilinoleate)/(lutein monopalmitate + lutein dipalmitate)*100. All samples were compared with a control, H35, which produced the same quantity of lutein esters with each fatty acid. Forty-six accessions favored the synthesis of lutein esters with palmitic acid (they are shown below the lower horizontal line) while three accessions (appearing above the upper horizontal line) produced a higher proportion of lutein esters with linoleic acid. The remaining forty-three produced similar amounts of lutein esters with each fatty acid at $P=0.05$, determined by two-sided Dunnett's multiple comparisons with H35 as control.

Fig. 8. Manhattan plots from MLM model for free lutein (a), lutein monolinoleate (b), lutein dipalmitate (c) and lutein linoleate-palmitate. For each trait, the LOD threshold determined using the Bonferroni correction at $P=0.05$ (LOD of 5.4).

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- Supplementary file list.**
- Supplementary file 1.** Alignment results of DArTSeq markers against barley genes.xlsx
- Supplementary file 2.** DArTSeq markers showing significant association after GWAS analysis.xlsx
- Supplementary file 3.** Comparison of the resulting groups in *H. chilense* with DArTseq markers and morphological characters.xlsx

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8