Universitat de Lleida

Analysis of the efficiency in the Spanish National Barley Breeding Program. Past results and prospects for future improvements using molecular markers

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CSIC
Consejo Superior de Investigaciones Científicas

Estación Experimental
de Aula Dei

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PhD

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Analysis of the efficiency in the Spanish National Barley Breeding Program. Past results and prospects for future improvements using molecular markers

Thesis presented by
Elsayed Mansour Elsayed
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of Lleida University

This work was carried out at the Department of Genetics and Plant Production, Aula Dei Experimental Station (EAAD), Spanish National Research Council (CSIC)

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UNIVERSITAT DE LLEIDA

Escola Técnica Superior d'Enginyeria Agrária

Memoria presentada por **Elsayed Mansour Elsayed**, Ingeniero Agrónomo, para optar al grado de Doctor Ingeniero Agrónomo por la Universidad de Lleida

El Doctorando

Elsayed Mansour Elsayed

AUTORIZACIÓN DE LOS DIRECTORES PARA LA PRESENTACIÓN DE TESIS DOCTORAL

D. ERNESTO IGARTUA ARREGUI, Investigador Científico del Consejo Superior de Investigaciones Científicas y Dª ANA MARÍA CASAS CENDOYA, Científico Titular del mismo organismo

INFORMAN:

Que la tesis doctoral titulada "**Analysis of the efficiency in the Spanish National Barley Breeding Program. Past results and prospects for future improvements using molecular markers**" ha sido realizada por el Ingeniero Agrónomo D. ELSAYED MANSOUR ELSAYED, en el Departamento de Genética y Producción Vegetal, de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su dirección y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor Ingeniero Agrónomo.

Zaragoza, Febrero de 2013

Fdo. Ernesto Igartua Arregui Fdo. Ana María Casas Cendoya

AUTORIZACIÓN DEL TUTOR ACADÉMICO PARA LA PRESENTACIÓN DE TESIS DOCTORAL

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Que la Tesis Doctoral titulada "**Analysis of the efficiency in the Spanish National Barley Breeding Program. Past results and prospects for future improvements using molecular markers**" ha sido realizada por el Ingeniero Agrónomo D. ELSAYED MANSOUR ELSAYED, en el Departamento de Genética y Producción Vegetal, de la Estación Experimental de Aula Dei del Consejo Superior de Investigaciones Científicas bajo su tutela y reúne, a su juicio, las condiciones requeridas para optar al Grado de Doctor Ingeniero Agrónomo

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To my parents and my wife

List of the abbreviations used

A1:	Albacete dry-land
A2:	Albacete irrigated
AMMI:	Additive main effect and multiplicative interaction model
ANOVA:	Analyses of variance
ARE:	Grain area
cM:	Centimorgan
DHE:	Days to heading
E:	Environment (combinations of years and locations)
G:	Genetic gain
GBS:	Genotyping-by-sequencing
GEI:	Genotype-by-environment interaction
GL:	Genotype-by-location
GLY:	Genotype-by-location-by-year
GRW:	Growth habit
GY:	Genotype-by-year
$h^{2:}$	Heritability
H:	Realized heritability
ha	Hectare
HEC:	Hectolitre weight
L:	Location
L1:	Artesa
L2:	Bell-lloc
L3:	Gimenells
L4:	Solsona
LEN:	Grain length
LY:	Location-by-year
MAS:	Marker assisted selection
MAT:	Maturity time
MET:	Multi Environment Trials
PCA:	Principal component axis
PHE :	Plant height
POW:	Powdery mildew
QTL:	Quantitative trait loci
REML:	Restricted maximum likelihood
RILs:	Recombinant inbred lines
S:	Selection differential
SNP:	Single nucleotide polymorphism
SSR:	Simple sequence repeats (Microsatellites)

SPO: Spot blotch
TGW: Thousand grain weight
V1: Valladolid capital
V2: Villahoz
V3: Ceinos
V5: Macotera
VIG: Early vigor
WID: Grain width
Y: Year
YLD: Grain yield
Z1: Sádaba
Z2: Vedado

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Shaded areas indicated the regions with selection QTL. Top graph, all markers; intermediate graph, markers with unique position; bottom graph, markers with multiple positions in the physical map.

Annex 6.5. Gracia MP, E Mansour, AM Casas, JM Lasa, B Medina, JL Molina-Cano, MA Moralejo, A López, P López-Fuster, J Escribano, FJ Ciudad, P Codesal, JL Montoya, E Igartua. 2012. Progress in the Spanish National Barley Breeding Program. Spanish Journal of Agricultural Research 10:741-751

Summary / Resumen / Resum

Summary

The overall objectives of this thesis were to test the efficiency of selection in the Spanish Barley Breeding Program and to find the most important genetic factors responsible for the advantage of elite material, to facilitate future selection.

The progress in the breeding program was estimated restrospectively, using data generated in trials of the advanced generations (F8, F9, F10) over a long series of years. Progress in the program was evident, with increasing yields in each generation, and with advanced lines surpassing the checks in the last two generations. Although the genotype by environment interaction (GEI) found for grain yield was quite large, it showed no apparent underlying geographic patterns. However, the results of some locations hinted that environmental causes might be causing GEI.

The relationship between GEI and climatic variables was investigated in more detail using again retrospective data of 11 years of advanced generations of the breeding program, across 12 locations. An in-depth analysis of the check cultivars revealed that one of the apparent causes of grain yield GEI was the occurrence of differential genotypic responses to winter temperatures. The analysis of the main lines tested in the program confirmed that genotypes having different vernalization requirements reacted differentially to winter temperatures, and that this had an impact on grain yield. These results highlight the importance of defining appropriate patterns of adaptation to the prevailing climate.

Quantitative trait loci analysis (QTLs) was done for a population of recombinant inbred lines (RILs) developed from one of the best crosses of the breeding program. The analysis of five field trials made possible the detection of thirty-three QTLs for agronomic traits. Some of them are proposed as future breeding targets for marker assisted selection (MAS) in the same type of crosses. The main vernalization gene in barley, *VrnH1* was detected as the main factor responsible for optimum adaptation and production of barley across a range of typically Mediterranean environments. A study of selection QTLs in the advanced lines of the program confirmed the large influence of the *VrnH1* region in the breeding process. Only a few selection QTLs confirmed the QTLs found in the RIL population but, on the other hand, provided evidence for strong selection at several genomic regions that may be targeted through MAS in the future.

Resumen

Los objetivos generales de esta tesis fueron evaluar la eficiencia de la selección en el Programa Nacional Español de Mejora de Cebada y encontrar los factores genéticos responsables de la ventaja del material elite, para facilitar la selección en el futuro.El progreso en el programa se estimó retrospectivamente, usando datos generados en los ensayos de las generaciones avanzadas (F8, F9, F10) a lo largo de una serie de años representativa. El progreso obtenido fue evidente, con rendimientos crecientes en cada generación, sobrepasando a los testigos en las dos últimas generaciones. Aunque se detectó una importante interacción genotipo-por ambiente (GEI) para el rendimiento, ésta no mostró patrones geográficos aparentes. Sin embargo, los resultados de algunas localidades indicaron que podría haber causas ambientales en la base de la GEI.

La relación entre la GEI y variables climáticas se investigó con más detalle utilizando los datos retrospectivos de 11 años de generaciones avanzadas del programa de mejora, correspondientes a 12 localidades. Un análisis de las variedades empleadas como testigos indicó la relación de la GEI con las diferentes respuestas de los genotipos a la temperatura invernal. El análisis de las líneas avanzadas del programa confirmó que los genotipos reaccionaron diferencialmente a las temperaturas de invierno según su necesidad de vernalización, y que esto afectó al rendimiento. Estos resultados mostraron la importancia de definir patrones apropiados de adaptación al clima imperante.

Se llevó a cabo un análisis de QTL con una población de líneas consanguíneas recombinantes (recombinant inbred lines, RIL), desarrollada a partir de uno de los mejores cruzamentos del programa de mejora. El análisis de cinco ensayos de campo permitió la detección de treinta y tres QTLs de caracteres agronómicos. Algunos de ellos se proponen como futuros objetivos de mejora para usar la selección asistida por marcadores (MAS) en los mismos tipos de cruzamentos. El gen principal de la vernalización en la cebada, *VrnH1* se reveló como el principal factor responsable de la óptima adaptación y producción de cebada en ambientes mediterráneos. Un estudio de QTLs de selección en las líneas avanzadas del programa confirmó la gran influencia de la región de *VrnH1* en el proceso de mejora. Los QTLs de selección detectados en las líneas avanzadas del programa confirmaron sólo algunos de los QTL encontrados en la población RIL pero, por otro lado, proporcionaron evidencia de la presencia de una fuerte selección en varias regiones genómicas, que también pueden ser objetivo de MAS en el futuro.

Resum

Els objectius generals d'aquesta tesi foren avaluar l'eficiència de la selecció al Programa Nacional Espanyol de Millora d'Ordi i trobar els factors genètics responsables de l'avantatge del material elit, per facilitar la selecció al futur. El progrés en el programa es va estimar retrospectivament, utilitzant dades generades als assajos de les generacions avançades (F8, F9, F10) al llarg d'una sèrie d'anys representativa. El progrés obtingut va ser evident, amb rendiments creixents a cada generació, sobrepassant els testimonis en les dues últimes generacions. Tot i que es va detectar una important interacció genotip-per ambient (GEH) per al rendiment, aquesta no va mostrar patrons geogràfics aparents. No obstant, els resultats d'algunes localitats han indicat que podria haver causes ambientals a la base de la GEH.

La relació entre la GEH i variables climàtiques es va investigar amb més detall utilitzant les dades retrospectives de 11 anys de generacions avançades del programa de millora, corresponents a 12 localitats. Una anàlisi de les varietats emprades com a testimonis va indicar la relació de la GEH amb les diferents respostes dels genotips a la temperatura hivernal. L'anàlisi de les línies avançades del programa va confirmar que els genotips van reaccionar diferencialment a les temperatures d'hivern segons la seva necessitat de vernalització, i que això va afectar el rendiment. Aquests resultats van mostrar la importància de definir patrons apropiats d'adaptació al clima imperant.

Es va dur a terme una anàlisi de QTL amb una població de línies consanguínies recombinants (recombinant inbred lines, RIL), desenvolupada a partir d'un dels millors creuaments del programa de millora. L'anàlisi de cinc assaigs de camp va permetre la detecció de trenta-tres QTLs de caràcters agronòmics. Alguns d'ells es proposen com a futurs objectius de millora per utilitzar la selecció assistida per marcadors (MAS) als mateixos tipus de creuaments. El gen principal de la vernalització en l'ordi, VrnH1 es va revelar com el principal factor responsable de l'òptima adaptació i producció d'ordi en ambients mediterranis. Un estudi de QTLs de selecció en les línies avançades del programa va confirmar la gran influència de la regió de VrnH1 en el procés de millora. Els QTLs de selecció detectats en les línies avançades del programa van confirmar només alguns dels QTL trobats a la població RIL però, d'altra banda, van proporcionar evidència de la presència d'una forta selecció en diverses regions genòmiques, que també poden ser objectiu de MAS en el futur.

Chapter 1

General Introduction

Chapter 1: General Introduction

1.1. Economic importance

Barley, *Hordeum vulgare* L., is an important cereal crop, ranking fourth in the world in terms of planted area after only wheat, rice and maize (Xue et al. 2010). It is one of the main cereals of Mediterranean agriculture, and a founder crop of Old World Neolithic food production. It was probably the first species cultivated as a food crop for human consumption (Baik and Ullrich 2008).

Barley is regarded as an inferior staple compared to wheat, and is considered as the poor people's bread. It is commonly grown under conditions inducing low productivity, such as dry conditions, poor soils and soil or water salinity, where it has a productive advantage over wheat. Because of these characteristics, it has been the principal grain produced in numerous stress-prone areas.

Barley was presumably first used as human food but later on evolved primarily into a feed, malting and brewing grain due in part to the rise in prominence of wheat and rice (Baik and Ullrich 2008). Historically, barley has been an important food source in many parts of the world, including the Middle East, North Africa and northern and eastern Europe (Iran, Morocco, Ethiopia, Finland, England, Denmark, Russia and Poland), and Asia (Japan, India, Tibet and Korea) (OECD 2004; Newman and Newman 2006). Food barley is generally found in regions where other cereals do not grow well due to altitude, low rainfall, or soil salinity.

The major use of barley today is mainly for livestock feed. Globally, up to 85% of barley produced is used for feeding animals, including cattle (beef and dairy), and poultry (Pickering and Johnston 2005; Setotaw et al. 2010). The second most important use of barley is for malt, which is used mostly in the making of beer and liquors, but is also a component in a variety of foods, such as biscuits, bread, cakes and desserts (Baik and Ullrich 2008). Barley has also minor uses in the pharmaceutical industry.

Economically, barley is a major commodity for most major European and North African countries. Therefore, many countries in these areas maintain active barley breeding programs. Spain has a public national barley breeding program, which can benefit from progresses in the knowledge about the genetic determinants of grain yield and other relevant agronomic traits.

Barley ranks as the fourth most important cereal in the world, after wheat, maize and rice (FAOSTAT 2010). The barley cultivation area and the production in Spain and Europe in the last 10 years are shown in Table 1 (FAOSTAT 2010).

Table 1.1. Barley cultivation area and production in Spain and Europe over the last 10 years.

Year	Spanish cultivation area (million ha)	Spanish production (million tons)	European cultivation area (million ha)	European production (million tons)
2000	3.28	11.06	27.58	84.02
2001	2.99	6.24	29.24	92.39
2002	3.10	8.36	29.13	91.30
2003	3.17	8.70	28.93	83.20
2004	3.18	10.64	28.90	96.51
2005	3.16	4.62	27.99	83.09
2006	3.20	8.14	29.73	88.85
2007	3.23	11.94	27.32	82.84
2008	3.46	11.26	29.21	105.37
2009	3.05	7.35	27.73	95.59
2010	2.88	8.16	22.95	73.49

1.2. Taxonomy and diversity

Barley, *Hordeum vulgare* L., belongs to the grass family *Poaceae*. The *Poaceae* is the largest family of monocotyledonous plants. The genus *Hordeum* L. comprises 32 species (Bothmer et al. 1991). The progenitor of barley, *H. vulgare* ssp. *spontaneum* (C. Koch) Tell, is considered to be a subspecies of cultivated barley, as both types cross readily. Its origin can be traced back to the Fertile Crescent region, though other origins have also been postulated (Molina-Cano et al. 2002). Cultivated barley is almost completely self-pollinated with predominantly cleistogamous flowering behaviour (Jain 1976).

There is a huge diversity of cultivated types, with hundreds of modern varieties and thousands of landraces, still grown or kept in germplasm banks. All cultivars have non-brittle rachis, which means that the spike stays intact after ripening and can be harvested and threshed by farmers. This is one of the main traits which suffered fixation in the process of domestication of the species, in contrast with wild barleys, in which rachis is always brittle. Non-brittleness in cultivated barley is governed by a mutation in either one of two tightly linked ‘brittle´ genes (*Btr1*, *Btr2*) (Takahashi 1972).

Cultivated barleys are commonly classified according to different agronomic or quality traits, such as growth habit, spike morphology, grain morphology, etc. One of the main classifications attends to the seasonal growth habit of the cultivars, for which three main types have been described: winter, spring and facultative. Winter barley is sown in autumn. It is tolerant to low temperature, it requires vernalization to promote flowering, and commonly displays a strong promotion to flowering in response to long days. Spring barley is essentially the opposite of the winter barley. It usually has minimal low temperature tolerance, does not require vernalization, and is insensitive to long photoperiods. Facultative barley actually represents a subclass of the winter growth habit, typically utilized to refer to genotypes that are as low temperature tolerant as winter varieties, but lack a vernalization requirement (von Zitzewitz et al. 2005). Several genes, that will be introduced later, underlie a complex genetic control of this trait. Another essential classification of barley cultivars is made attending to spike morphology. According to this, barley can be divided into two-rowed and six-rowed types, though intermediate types also exist. In two-row barley, the lateral spikelets are sterile, whereas in six-row barley all spikelets are fertile. There are two main genes controlling spike type, *Vrs1* (Komatsuda et al. 2007) and *Int-c* (Ramsay et al. 2011).

1. 3. Cytology and Genetics

Barley is a diploid species with a low number of chromosomes (2n = 2x = 14). Barley is predominantly a self-pollinated crop. The seven chromosomes, identified and labeled based on their size and characteristics, are denominated 1H through 7H (Linde-Laursen et al. 1997). Its genome presents high homeology to wheat genomes A, B and D, and to the genomes of other grasses, allowing localization of chromosomal segments through synteny across species (Mayer et al. 2011).

Genomics in the Triticeae lagged behind other plant species, hampered by the large size (17 Gb for the bread wheat genome, i.e., 40x the rice genome; 5 Gb for barley and 8 Mb for rye) and complexity (high repeat content, polyploidy) of their genomes (Sreenivasulu et al. 2008; Close et al. 2009). Barley contains approximately 26,000 genes (International Barley Genome Sequencing Consortium, IBSC 2012). Comprehensive resources have been developed in barley, including largest sets of expressed sequence tags (ESTs), bacterial artificial chromosome (BAC) libraries, and DNA arrays (Varshney et al. 2007).

Bacterial artificial chromosome (BAC) libraries are large DNA insert libraries of choice and an indispensible tool for map based cloning, physical mapping, molecular cytogenetics, comparative genomics and genome sequencing. BAC libraries representing more than 20 haploid genomes as a new resource to the barley research community have been constructed (Schulte et al. 2011).

Genotyping-by-sequencing (GBS) has been developed as a tool for QTL for linkage and association studies and genomics-assisted breeding in a range of species including those with complex genomes. GBS uses restriction enzymes for targeted complexity reduction followed by multiplex sequencing to produce high-quality polymorphism data at a relatively low per sample cost (Poland and Rife 2012; Poland et al. 2012).

High-throughput genotyping platforms (Illumina SNP and DArT) have also been implemented in barley. This will increase the identification of marker trait associations, and the subsequent identification of potential candidate genes (Sreenivasulu et al. 2008; Comadran et al. 2012). Several technology developments during the last years have led to the development of a ‘‘Genomic toolbox’’ with new and more efficient resources that support the establishment of robust genomic programs in the Triticeae (Feuillet and Muehlbauer 2009).

Genomics can provide support for crop improvement by extending the amount or nature of variation available for selection, by allowing a precise transfer of traits reducing linkage drag, or by accelerating the selection process to produce varieties more rapidly. Essentially, the various -omics platforms improve the ability to discover genes and pathways that control specific traits and provide screening and analysis platforms to support selection strategies (Langridge and Fleury 2011).

Barley is highly autogamous, has a long history of recombination events and conserved linkage disequilibrium at the cM scale (Caldwell et al. 2006). This means that fewer markers are required to survey the whole genome compared to outbreeding species such as maize (Remington et al. 2001).

Over forty years ago, linkage data were available for only 79 loci in barley (Nilan 1964). Since then, there has been steady progress in building more and more dense linkage maps. Marker systems are increasingly gene-based, with the most recently published high-density map having 1032 expressed sequence tag (EST)-based loci (Stein et al. 2007). A 3000-EST locus map and a consensus, single nucleotide

polymorphism (SNP) map with 2943 loci are available at HarvEST (www.harvest-web.org; Muñoz-Amatriaín et al. 2011).

Additionally, the integration of genomic data into genebank documentation systems and its combination with taxonomic, phenotypic and ecological data will usher in a new era for the valorization of plant genetic resources (PGR) (Kilian and Graner 2012).

The access to important genomic resources is facilitating greatly the search for candidate genes. The last and most important resource recently made available to the research community is the access to an almost complete barley genome sequence. Though it still has some gaps, it is a very complete tool with an integrated and ordered physical, genetic and functional sequence resource that describes the barley gene-space in a structured whole-genome context (IBSC 2012).

1.4. Quantitative trait loci (QTLs) analysis

Many agriculturally important traits such as yield are controlled by numerous genes and are commonly known as quantitative traits (also 'polygenic', 'multifactorial' or 'complex' traits). The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTL, Paterson et al. 1991). Since the development of molecular markers, it has become feasible to identify and localize genetically the underlying polygenes as QTLs and to utilize these QTLs for crop improvement (Bernardo 2008; Xu and Crouch 2008).

The general goals of QTL mapping in plants are, on one hand, to increase the biological knowledge of the inheritance and genetic architecture of quantitative traits and, on the other hand, to identify markers that can be used as indirect selection tools in breeding (Bernardo 2008). In the last two decades, the ability to transfer target genomic regions using molecular markers resulted in extensive QTL mapping experiments in most crops economically important, aiming at the development of molecular markers for marker assisted selection (Xu 2010).

The use of molecular markers associated with these traits can greatly improve selection efficiency by circumventing environmental effects (Wang et al. 2010). The knowledge regarding QTL has led to remarkable advances in breeding for a variety of traits, some of which have an effect on yield under particular environmental conditions (Peighambari et al. 2005; Cuesta-Marcos et al. 2009). QTL analysis has facilitated the tracking of traits across environments in data collected from multiple environmental

trials (Xing et al. 2002). Also, they have spurred a revival of backcross procedures in breeding, because the precision of the transfer of genomic regions reduces linkage drag, a huge problem when using exotic germplasm sources (Tanksley and Nelson 1996; Pillen et al. 2004; Bauer et al. 2009).

The identification of QTLs based only on conventional phenotypic evaluation is not possible. The major breakthrough that made possible the identification of QTLs was the development of DNA (or molecular) markers in the 1980s (Guo and Nelson 2008). QTL identification consists of four components: a segregating population, segregating markers, phenotypic values for the individuals from measurement of trait(s) of interest and association of the phenotypic data for the trait with genotypic data using an appropriate statistical approach. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers.

QTL are identified using statistical procedures that integrate genotypic and phenotypic data and are attributed to regions of the genome at specified levels of statistical probability. Thus, mapping QTL is not as simple as mapping a gene that affects a qualitative trait (Semagn et al. 2010). The conventional methods for QTL mapping in plants include first generating a population [F2, backcross (BC), recombinant inbred lines (RIL) or doubled haploid (DH)] from a biparental cross, genotyping the individuals with genetic markers across the genome, phenotyping the individuals for the trait of interest, and then analyzing the results via linkage mapping (Flint-Garcia et al. 2005).

Progress in high throughput molecular marker platforms providing good genome coverage (from hundreds to thousands) together with decreasing genotyping costs have awakened the interests of plant geneticists in using naturally occurring variation for identifying genomic regions involved in complex traits (Close et al. 2009). The numbers of molecular markers for crop plants such as barley has increased and their cost has decreased therefore the number of QTL studies have increased exponentially (Rae et al. 2007).

1.4.1. Genetic mapping

Genetic mapping (also known as linkage mapping) is one of the various applications of molecular markers in any species. It refers to the determination of the relative positions of genes on a DNA molecule (chromosome) and of their distance between them. In genetics, the distance between genes on the genome is assessed on the

basis of the frequency of recombination of the genes, estimated from scoring genotypes of progeny of a cross (Kearsey and Pooni 1996). The recombination is first estimated for all markers that are segregating as expected, and then any marker that is linked to any other marker is placed in the same linkage group (Young 1996; Yin et al. 2003). The linear arrangement of markers into linkage groups, or chromosomes, provides the genetic map for locating QTL that are relative to intervals of markers (or statistically related sets of markers) (Doerge 2002).

Genetic map indicates the position and relative genetic distances between markers along chromosomes, which is analogous to signs or landmarks along a highway where the genes are "houses" (Collard et al. 2005). It places molecular genetic markers in linkage groups based on their co-segregation in a population. And predicts the linear arrangement of markers on a chromosome and maps are prepared by analysing populations derived from crosses of genetically diverse parents, and estimating the recombination frequency between genetic loci (Duran et al. 2009a). A genetic map provides a genetic representation of the chromosome on which the markers and QTL reside.

The genetic map can be used to localize QTL for a quantitative trait, as first demonstrated by Paterson et al. (1988). The construction of detailed genetic maps with high levels of genome allow detailed genetic analysis of qualitative and quantitative traits that enable localization of genes or quantitative trait loci (QTL) and facilitate the introgression of desirable genes or QTLs through marker-assisted selection (Yim et al. 2002). And also allow comparative mapping between different species in order to evaluate similarity between gene orders and function in the expression of a phenotype (Paterson et al. 2000).

QTL mapping was first described by Sax (1923), single marker analysis was used to detect a QTL in the vicinity of a marker by studying genetic markers individually. The approach is based on classifying the offspring into one of two classes depending on their genotype at the marker, calculating the mean trait value associated with each class of offspring and comparing the mean trait values for each class to get significant differences (Hackett 2002). Single point analysis does not require a complete molecular linkage map. But this analysis has some drawbacks such as labor requirement, decreased power to detect a QTL between markers and inability to distinguish between tight linkage to a QTL with small effect and loose linkage to a QTL with large effect (Collard et al. 2005).

Interval mapping is another approach for QTL analysis made popular by Lander and Botstein (1989), which uses an estimated genetic map as the framework for the location of QTL. The principle behind interval mapping is to test a model for presence of a QTL at many positions between two mapped marker loci. This model uses method of maximum likelihood or regression.

The method of composite interval mapping (CIM) has become popular for mapping QTLs and it was proposed as solution to SIM drawbacks. This method combines interval mapping with linear regression and includes additional genetic markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping. The main advantage of CIM is that it is more precise and effective at mapping QTLs compared to single-point analysis and interval mapping, especially when linked QTLs are involved (Zeng 1994).

1.4.2. Molecular markers

Molecular markers represent one of the most powerful tools for the analysis of genomes and enable the association of heritable traits with underlying genomic variation (Duran et al. 2009a). They arise from different classes of DNA mutations such as substitutions (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Paterson 1996).

The environments have no effect on DNA level or structure, therefore DNA based molecular markers are more widely used than other markers types. A wide variety of techniques can be used to detect DNA variations (Collard et al. 2005).

Molecular markers can be classified into three categories: hybridization-based DNA markers such as RFLP; PCR-based DNA markers such as RAPD, SCAR, STS, SSR and AFLP, and DNA chip-based microarray such as SNP (Winter and Kahl 1995).

Single nucleotide polymorphism (SNP) markers represent just a single base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position. To be considered as an SNP, the least frequent allele should have a frequency of 1% or greater. Although in principle, at each position of a sequence stretch, any of the four possible nucleotide bases can be present, SNPs are usually biallelic in practice (Vignal et al. 2002). There are three different forms of SNP, transitions (C/T or G/A), transversions (C/G, A/T, C/A or T/G) and small insertions–deletions (indels) (Duran et al. 2009b).

The development of high-throughput methods for the detection of single nucleotide polymorphisms (SNPs) and small indels (insertion/deletions) has led to a revolution in their use as molecular markers. SNPs are increasingly becoming the marker of choice in genetic analysis and are used routinely as markers in agricultural breeding programs (Gupta et al. 2001).

SNPs have many applications in plant genetic studies. These include high-resolution genetic map construction (Rafalski 2002), diversity studies (Kilian and Graner 2012) or even gene identification (Comadran et al. 2012). The use of SNPs is becoming widespread with the increasing availability of crop genome sequence, the reduction in cost, and the increased throughput of SNP assays (Batley et al. 2007).

1.4.3. Mapping populations

There are different kinds of populations can be used effectively for QTL mapping. F2 populations are developed by selfing F1 individuals, which are developed from crossing two (usually) homozygous parents. Crossing F1 individuals with one of the parents develops backcross populations (Paterson 1996). Recombinant inbred lines (RILs) are formed by crossing two genotypes followed by repeated selfing to create a new set of inbred lines whose genome is a mosaic of the parental genomes (Broman 2005). And doubled haploid (DH) populations are produced by generating plants by anther or microspore culture followed by chromosome doubling (Thompson et al. 1991).

Each RIL and DH is an inbred line, and so can be propagated eternally. A panel of lines of this kind has a number of advantages for genetic mapping: one needs to genotype each line only once; one can phenotype multiple individuals from each line to reduce individual, environmental, and measurement variability; multiple phenotypes can be obtained on the same set of genomes. An additional advantage of RILs over DH is that, recombination is richer because the breakpoints in RILs are denser due to the occurrence of a larger number of meiosis compared with populations in which only one meiosis takes place, as is the case for DH, and greater mapping resolution can be achieved (Broman 2005).

The choice of mapping population type depends on the crop species, and on the marker system used. Each type of population will give a specific segregation ratio at each locus. In an F2, dominant and co-dominant markers segregate 3:1 and 1:2:1,

respectively, while the segregation of both marker types is 1:1 in BC, DH and RIL. Using F2 can maximize the information of co-dominant markers, using DH or RIL can maximize the information obtained by dominant markers. BC and F2 are not eternal; therefore the source of tissue for DNA or protein is limited. Both DH and RIL populations can produce hundreds of identical seeds so that unchanging genotypes can be evaluated repeatedly over years and locations in multiple traits (Burr et al. 1988).

1.5. Genotype × Environment interaction

The aim of plant breeding is to create new genotypes with higher yield, and stable under various conditions of cultivation, particularly under conditions which are less favourable for plant growth and development (Arshad et al. 2003). Genotype by environment interaction (GEI) is said to occur when cultivars or genotypes respond differently to diverse environments (Yan and Kang 2003).

High GEI mean that genotypes grown in multienvironmental trials tend to react substantially differently to varying environmental conditions (Comadran et al. 2011). Gauch and Zobel (1996) explained the importance of GEI as: "Were there no interaction, a single variety would yield the most the world over, and furthermore the variety trial need to be conducted at only one location to provide universal results. And were there no noise, experimental results would be exact, identifying the best variety without error, and there would be no need for replication. So, one replicate at one location would identify that one best variety that flourishes worldwide".

Plant breeders and geneticists, as well as statisticians, have a long-standing interest in investigating and integrating the genotypic effect (G) and Genotype by environment interaction effect (GEI), as the latter seriously impairs efforts in selecting superior genotypes relative to new crop introductions and cultivar development programs (Yan et al. 2000). The decisions about the commercial value of new crop varieties are usually based on data from Multi Environment Trials (MET) series, done over several locations and years, across the target environment (Smith et al. 2001). MET series are designed to cover the range of agro-ecological conditions that may occur in the target environment (Romagosa et al. 2009), and thus provide an unbiased set of data to support the selection process.

The basic model that includes GEI is:

$$P_{ij} = \mu + G_i + E_j + GE_{ij}$$

Here, P_{ij} is the measured yield of each cultivar at each testing environment, is the result of adding μ, the overall mean, an environment main effect (E_j), a genotype main effect (G_i), and the genotype by environment interaction(GE^{ij}) (Yan and Kang 2003).

Understanding of the causes of GEI is important at all stages of plant breeding. It affects ideotype design, parent selection, and selection based on yield or other traits. It can help to identify traits that contribute to better cultivar performance and environments that facilitate cultivar evaluation. And also could be used to establish breeding objectives, identify ideal test conditions, and formulate recommendations for areas of optimal cultivar adaptation (Yan et al. 2001).

In the last decade, efforts to elucidate the genetic factors causing GEI have veered towards the use of molecular markers. Quantitative trait loci responsible for adaptation have been reported in several populations (Romagosa et al. 1996; Bezant et al. 1997; Zhu et al. 1999; Lanceras et al. 2004; Maccaferri et al. 2008). Zheng et al. (2010) have illustrated the identification of QTL specific for certain environments by the combined use of a set of probe genotypes to characterize 12 environments (in terms of water deficit, radiation, temperature or nitrogen stress) and the analysis of a wheat mapping population. In that study, genotype and QTL by environment interactions were partitioned using environmental covariates for those environments where kernel number and thousand kernel weight QTL were identified.

Identification of QTL is useful to explain the genetic regulation of phenotypes and may provide markers that can assist in plant breeding. However, many QTL studies have produced inconsistent results regarding their detection in different environments (Leflon et al. 2005), as a result of the presence of GEI. Therefore, understanding the genetic basis of the GEI is a key objective to find the genetic factors underlying adaptation of genotypes to specific environments (Zheng et al. 2010). The study of GEI using conventional biometrical procedures has benefited greatly from the development of molecular markers to measure individual genetic effects and dissect GEI into QTL × environment interactions (Emebiri and Moody 2006).

Several studies have conducted multi-environment trials for various traits in different plant species, including grain yield in barley (Romagosa et al. 1996; Teulat et al. 2001; Voltas et al. 2001; Malosetti et al. 2004). They all succeeded in identifying loci that interacted with the environment, i.e. loci underlying GEI. Some loci for GEI

co-localized with loci for the trait mean expression, whereas others appeared at positions where no QTLs for the mean expression were found.

1.6. Flowering date

The transition from vegetative to reproductive growth is a critical developmental switch and a key adaptive trait in both crop and wild cereal species, because it ensures that plants set their flowers at an optimum time for pollination, seed development, and dispersal (Cockram et al. 2007). This transition is often difficult to see unless the plants are dissected. Thus, surrogate traits easily recordable by naked eye are used to monitor the advancement of plant growth. One of these traits is the date of flowering, also regarded as one of the most important adaptive characteristics of plants (Laurie 2009). At flowering, most of the newly produced carbohydrates are transported to the developing seed and resources accumulated in storage tissues during the vegetative growth phase are reallocated to the production of seeds (Brachi et al. 2010).

In cereals, as in many other species, the timing of this transition, commonly known as transition to flowering, is determined by seasonal changes that are sensed by the plant (Sung and Amasino 2004). The successful sexual reproduction in plants and ensuing development of seeds depends on flowering at the right time, therefore the maximum yield attainable in a growing season is determined during the pre-flowering period (Slafer 2003). Mechanisms that control flowering in response to environmental stimuli such as day length (photoperiod) and periods of low temperature (vernalization) are important adaptive factors and have major impacts on agriculture (Dunford el al. 2005).

Flowering time is a complex trait shaped by selective pressures acting on very different spatial scales (Brachi et al. 2010).Temperature and photoperiod are the two major environmental factors that affect time to flowering in annual species like cereals, particularly those whose growing season includes the winter (Loomis and Connor 1992; Laurie et al. 2004). Temperate environments with a long growing season allow cereal crops to flower late in the year and thus exploit an extended vegetative period for resource storage. Conversely, early flowering has evolved as an adaptation to short growing seasons. Depending on the climatic conditions of the region, barley sowings can be made in autumn, to take advantage of a longer season, or in winter or spring, to make full use of mild springs and summers and to escape winters that are too cold. Knowingly, or unknowingly, farmers and plant breeders have selected differences in

flowering date to increase yield and extend the agricultural flexibility and ecogeographical range of crops (Cockram et al. 2007).

Therefore flowering date has been an important trait for improving crop productivity and adaptation (Lawn et al. 1995), and is a primary objective of all breeding programs around the world. The genetics and physiology of heading date have been investigated by many researchers over many years. This is also true for barley and other temperate cereals, in which flowering date is a highly variable phenotypic trait which major implications for adaptation to geographic regions and crop management practices (Slafer 2003). In Mediterranean environments flowering date is considered a key trait for the adaptation of barley because barley is often grown under semi-arid conditions. Therefore, barley breeding programs must include the objective of achieving an appropriate flowering date among their targets (Cuesta-Marcos et al. 2009).

The major genes that control flowering time in barley in response to environmental cues are *VrnH1*, *VrnH2*, *VrnH3*, *PpdH1*, and *PpdH2* (Kikuchi et al. 2009; Casao et al. 2011a). All have been cloned in recent years (Yan et al. 2003, 2004, 2006; Trevaskis et al. 2003; Turner et al. 2005; Faure et al. 2007; Kikuchi et al. 2009). The series of *Vrn* genes have been mapped: *VrnH1* on chromosome 5H and *VrnH2* on 4H (von Zitzewitz et al. 2005), and *VrnH3* on 7H (Yan et al. 2006). The major loci affecting the photoperiod response, first identified as QTL were mapped to the long arm of chromosome 1H (*PpdH2*) and to the short arm of the 2H (*PpdH1*) by Laurie et al. (1995). Dominant alleles at *PpdH1* confer early flowering under long days, but have no effect under short days, whereas for *PpdH2* the dominant allele confers earliness under short days (Laurie et al. 1995), although a more general effect in winter cultivars has been proposed recently (Casao et al. 2011b). This locus provides an extra boost towards flowering for cultivars whose growth season occurs mostly under short days, particularly when vernalization is not complete.

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Chapter 2

Objectives

Chapter 2: Objectives

The main objectives of the thesis were:

1. To evaluate the effectiveness of the Spanish National Barley Breeding Program retrospectively, to estimate the progress achieved in grain yield in the advanced generations (F8-F10), and to assess the extent and the impact of genotype-by-environment interaction on grain yield.

2. To study the relationship between genotype-by-environment interaction of grain yield and environmental features. In the case of temperature, to explore if the interaction is affected by the growth habit of the cultivars, and to determine if genotype-by-environment interaction is related to climatic variation among the trials.

3. To detect quantitative trait loci (QTL) for agronomic traits relevant for Mediterranean conditions in an elite population, and to investigate the genetic factors that underlie the advantageous traits found in this population to facilitate the design of new breeding strategies and the implementation of marker assisted selection for Mediterranean conditions.

4. To detect selection QTLs through the retrospective study of the effect of selection on allelic frequencies across genomic regions affected by selection, indicating further possible targets for performing marker assisted selection.

Chapter 3

Progress in the Spanish National Barley Breeding Program

3.1. Introduction

Barley, *Hordeum vulgare* L., is one of the most important cereal crops in the world (Baik and Ullrich 2008), and it is grown in regions with climates unfavorable for production of other major cereals. It is commonly grown under dry conditions, poor and even saline soils, where it has a productive advantage. Because of these characteristics, it has been the main grain produced in numerous stress-prone areas (Poehlman 1985; Guttier et al. 2001), including the Mediterranean basin. In 2010, the barley cultivation area in Spain was 2.88 million hectares, and the production was 8.16 million tons, which corresponded to 23% of the total area devoted to barley in the European Union, and 15.3% of the total production (FAOSTAT 2012). It is the first crop in terms of acreage in Spain, being mostly grown in dry inland areas.

Despite being such an important crop for Spain, the breeding activities carried out by private companies are almost non-existent. The reason is the low profit obtained from sales of seed, as less than 10% of the surface is sown to certified seed. As a consequence, most cultivars available to growers in Spain have been bred in other countries. Even though some of these cultivars perform quite well in Spain, we expect that local breeding should result in superior cultivars. Studies carried out in the Mediterranean region have demonstrated that the most effective way to improve productivity of crops grown in less-favored areas is to use locally adapted germplasm and select in the target environment(s) (Ceccarelli 1994; Ceccarelli et al. 1998). The Spanish program takes advantage of this approach by local testing and also by the use of local landraces (Lasa 2008) as source of adaptation traits.

Therefore, there was a need to provide Spanish growers with cultivars adapted to their local conditions. The Spanish National Barley Breeding Programwas set out by four public research organizations with this purpose. These four centres are placed at the most representative barley growing regions of Spain. The program is conducted in a joint manner by four public research bodies: Instituto Técnico Agronómico Provincial (ITAP) in Albacete, Instituto de Investigación y Tecnología Agroalimentarias (IRTA) in Lleida, Instituto Tecnológico Agrario de Castilla y León (ITACyL) in Valladolid and Estación Experimental de Aula Dei (EEAD-CSIC) in Zaragoza (Fig. 3.1).

The main objectives of this study were to study the progress and the selection efficiency in the Spanish National Barley Breeding Program, and to verify if this progress occurred uniformly across the four provinces of the program. Also, we wanted

to have a general assessment of the extent and impact of genotype-by-environment interaction (GEI) of grain yield in the final stages of the program. This study will focus on grain yield, the main target of the breeding program, but also on its relationship with flowering date. Flowering date is one of the most important traits for improving crop productivity and adaptation (Lawn et al. 1995; Laurie 2009; Brachi et al. 2010), and is a primary objective of all breeding programs around the world.

Figure 3.1. Location of the testing sites of the Spanish National Barley Breeding Program. Provinces (in grey) and locations (in black) hosting field trials.

3.2. Material and methods

Program description

The breeding program follows a strict pedigree scheme. Lines are extracted from the F2, and advanced up to the F10 following a head-row system. Early generation testing takes place from F3 up to F5, independently at each site. F6 is the first generation of joint testing where the lines from the four provinces are merged together for testing. The advanced trials start in F7 and continue up to F10. The number of lines selected is reduced at each generation.

At each province, several locations were used for testing (Fig. 3.1). In Albacete two trials were carried out in the same location: Albacete dry-land (A1) and Albacete irrigated (A2). In Lleida, four locations were used: Artesa (L1), Bell-lloc (L2), Gimenells (L3) and Solsona (L4). In Valladolid, several locations were used: Castronuevo (V1), Geria (V1), Villabañez (V1), Zamadueñas (V1), Villahoz (V2), Ceinos (V3), La Espina (V4) and Macotera (V5). Four locations near the capital city of Valladolid were used in different years. These locations were close enough to each other to be considered as a single location, V1. And in Zaragoza two locations were used: Sádaba (Z1), Vedado (Z2). For two years, a location from a neighboring province, Navarra, was used. This was coded as Z3, since it was close to the locations from Zaragoza (Fig. 3.1). Not all locations were used every year. Trials were rotated between locations, with the exception of Albacete, and Zaragoza. There were two trials grown per province and year.

All the locations under study are non-irrigated locations, except Gimenells (L3), where irrigation was provided as needed to avoid losing the trial when drought was severe, and Albacete irrigated (A2), which was always under irrigation.

The temperature in the locations under study shows patterns typical of the Mediterranean climate, but with some differences from location to location (Fig. 3.2). Long term averages for temperature values were collected from the nearest meteorological stations to the locations under study (Table 3.1).

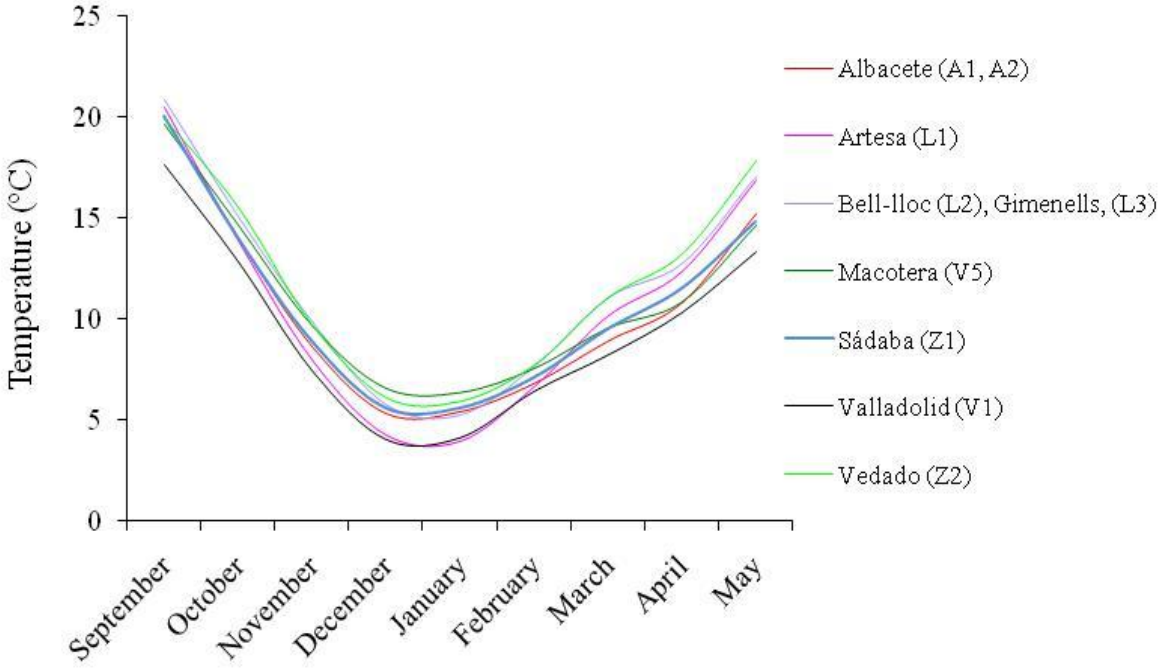

Figure 3.2. Long term monthly average temperatures for the testing locations.

Table 3.1. Coordinates of the testing locations of the Spanish National Barley Breeding Program and nearby meteorological stations used to collect long term climatic data.

Location	Latitude	Longitude	Meteorological station	Latitude	Longitude
Albacete (A1, A2)	38°59′N	1°51′W	Albacete	38°59′N	1°51′W
Artesa (L1)	41°33′N	0°42′E	Agramunt (Lleida)	41°47′N	1°06′E
Bell-lloc (L2)	41°37′N	0°46′E	Almacelles (Lleida)	41°43′N	0°26′E
Gimenells (L3)	41°39′N	0°23′E	Almacelles (Lleida)	41° 43′N	0° 26′E
Solsona (L4)	41°59′N	1°31′E			
Valladolid (V1)	41°38′N	4°43′W	San Miguel del Pino (Valladolid)	41°30′N	4°54′W
Villahoz[1] (V2)	42°04′N	3°54′W			
Ceinos (V3)	42°02′N	5°09′W			
La Espina (V4)	43° 23′N	6° 20′W			
Macotera[1] (V5)	40°49′N	5°17′W	Aldeaseca de Alba (Salamanca)	40°49′N	5°26′W
Sádaba (Z1)	42°17′N	1°16′W	Luna (Zaragoza)	42°10′N	0°52′W
Vedado (Z2)	41°51′N	0°39′W	EEAD-CSIC	41°43′N	0°48′W
Navarra[1] (Z3)	42°49'N	1°38'W			

[1] In some cases, locations from neighbouring provinces were used, but they were grouped together with the locations of each reference province.

Data set

The data of this study were collected from the advanced stages of the Spanish National Barley Breeding Program. The analysis focuses on the advanced generations of the program, with a low number of lines per generation (Table 3.2). In these advanced trials, grain yield was the main selection criterion. The data set was gathered from 163 trials corresponding to generations F8, F9 and F10 carried out from 1998 until 2008. A total of 349 advanced lines were studied during that period. Out of these, 327 were recombinant inbred lines derived from 197 hybridizations, and 22 were double haploid lines. Besides, up to 24 check varieties were evaluated in the trials (Table 3.2).

Table 3.2. Summary of lines and checks used in the advanced generations trials at the Spanish Barley Breeding Program.

Years	Common checks	F8	F9	F10	F8	F9	F10
		Additional checks			Test lines		
1998	Barbarrosa, Alpha, Zaida	5	2	7	25	15	14
1999	Barbarrosa, Alpha, Zaida	2	2	6	20	11	7
2000	Barbarrosa, Alpha, Zaida, Graphic	1	6	6	23	4	4
2001	Barbarrosa, Alpha, Zaida, Graphic	2	6	6	30	6	1
2002	Barbarrosa, Alpha, Zaida, Graphic	1	1	2	23	15	6
2003	Barbarrosa, Alpha, Zaida, Graphic	0	1	2	32	15	12
2004	Barbarrosa, Alpha, Zaida, Graphic, Hispanic	0	0	0	31	15	11
2005	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	14
2006	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	11
2007	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	11
2008	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	10
Total					312	145	101

The trials of the advanced generations followed an alpha-lattice of variable block size, with three replications, embedded in a randomized complete block design, with several test lines and checks. Each plot occupied 7.2 m^2 (6 m × 1.2 m), with either 6 or 8 rows. This area was modified for this study to 10.5 m^2 (7 m × 1.5 m) to take into account border effects.

The traits considered were raw grain yield (in kg ha^{-1}) at 10% moisture; relative grain yield for each line, expressed as the percentage of the average grain yield of the checks present at each particular trial; and flowering date, recorded as number of days

from January 1st when at least 2 cm of the awns were visible in 50% of the tillers of each plot.

The use of relative grain yield allows homogenizing the results among years and locations, and among analyses, therefore avoiding possible problems of scale due to differences in productivity across years and locations.

The data set is highly unbalanced because it was collected over 11 years, and the maximum period that any line stayed in the program was for three years. The advanced lines stayed in the program 1, 2 or 3 years, depending on the generation in which they were discarded. There were a few exceptions because some lines were introduced directly either in F9 or F10. For these lines, previous generations are missing. Also, a few lines were retained for additional years after F10, to get additional data before a final decision was made. To cope with the unbalancedness of the data, a mixed model approach (REML) was used, implemented in the software package Genstat 12 (Payne et al. 2009).

The relative grain yield was used to estimate the progress in the Spanish National Barley Breeding Program. To calculate the averages for each generation at each main location and province, two separate analyses were calculated using mixed models, considering locations or provinces as fixed factors, whereas years and the interactions with years were considered as random factors.

To calculate selection differential, genetic gain and realized heritability, the procedure of St. Martin and McBlain (1991) was used. The procedure is a test in which a set of lines evaluated in a generation is paired with a test in the next stage, in which selections from the set are re-evaluated. The procedure was adjusted to allow for the presence of different checks in the consecutive generations, which occurred in our data in some occasions. These calculations were done for the two selection steps available: F8-F9 and F9-F10, according to these expressions:

$$S = (X_s - X) \bullet 100$$
$$G = (X'_s - X) \bullet 100$$
$$H = G/S \bullet 100$$

where S is Selection differential, X_s is the mean of the experimental lines selected from the first stage (F8 or F9) for testing in the successive second stage (F9 or F10), X is the mean of all experimental lines evaluated in the first stage (F8 or F9), G is Genetic gain,

X'_s is the mean of the experimental lines selected from the first stage and evaluated in second stage (F9 or F10) and H is the realized heritability.

To calculate the components of variance, the complete data set was used, but divided into two groups, according to the presence of a minimum of three common checks among the trials. The first group contained 242 genotypes and 12 locations during 7 years (1998-2004) and the second group contained 163 genotypes and 11 locations, during 4 years (2005-2008), with some genotypes represented in the two analyses. Even though the data were unbalanced, the presence of a minimum of common checks in all trials of each group of years, plus the presence of some breeding lines for two or three consecutive years, provided enough replication of genotypes to allow an estimation of variance components.

The components of variance were calculated using the original raw grain yield data. Genotypic averages per locations were used for these analyses, as these are the data available for all trials. For the sake of this analysis, genotypes, locations and years were considered as random factors, as they can be regarded as random samples of all possible levels of each factor that can be encountered for barley growing in Spain.

To break-down the GEI into 'Genotype × Province' and 'Genotype within Provinces' interaction, two homogeneous series of genotypes repeated for two years were identified, *i.e.* 1998-1999, 2001-2002, 2003-2004, 2005-2006, and 2007-2008. Each series contained a group of genotypes tested in the same environments (combinations of years and locations) at two consecutive years. Analyses of variance (ANOVA) for relative grain yield were calculated for two series of balanced groups of genotypes. The first series contains the groups of lines in generations F8 and F9 at two consecutive years. And the second series contains groups of lines in generations F9 and F10 at two consecutive years. Each series contains five groups.

Linear regression was used to calculate the regression coefficient between flowering date and relative grain yield using the appropriate routine in Genstat 12.

3.3 Results

In all the advanced trials (F8, F9 and F10), several outstanding cultivars were included as checks. The number of checks varied from year to year, and also between locations, especially during the first years (Table 3.2). The checks were gradually changed along the years, always aiming to include the best cultivars available, combining spring and winter cultivars. A set of common checks was maintained across locations, ranging from 3 to 5 checks per year. These common checks were chosen because they were used in the national trials for cultivar registration, and kept shifting as these cultivars were being renewed.

The selection pressure applied from generation to generation was not constant across years and, overall, was stronger at F8 (46% of lines promoted to F9) than at F9 (70% of lines promoted to F10).

The number of lines tested varied among years, with an average of 28, 13, and 9 lines tested in F8, F9, and F10, respectively (Table 3.2). In the period under study, a minimum of 31 genotypes were evaluated every year at advanced trials. Over the years, the program has become more stable in terms of number of checks and lines under test at every generation.

In the data set under study there was a large range in the grain yields recorded, from a minimum of 842 kg ha^{-1} to a maximum of 6974 kg ha^{-1}. The overall mean for the entire period was 3687 kg ha^{-1}. The productivity levels were quite different between locations. The least productive location was Albacete dry-land (A1). The highest yielding location was Bell-lloc (L2). Productivity was also high in Gimenells (L3), Albacete irrigated (A2) and Macotera (V5), intermediate in Ceinos (V3), V1 (Castronuevo, Geria, Villabañez and Zamadueñas), Sádaba (Z1), Vedado (Z2) and Artesa (L1) (Table 3.3).

Table 3.3. Grain yield expressed as percentage of checks and average productivity in different locations and provinces, in the last three generations (F8, F9 and F10) of the Spanish Barley Breeding Program from 1998 to 2008. Averages across provinces and overall average, calculated with REML, in bold type.

	F8	F9	F10	Grain yield (kg ha^{-1})
A1	96.3	101.4	96.5	2683
A2	98.1	101.4	105.9	4517
Albacete	**96.0**	**100.7**	**100.8**	**3626**
L1	101.2	101.9	102.5	3012
L2	102.3	107.3	107.4	4966
L3	99.4	94.5	98.4	4636
Lleida	**101.1**	**101.3**	**102.8**	**4179**
V1	99.0	100.8	97.4	3478
V3	94.9	106.4	102.9	3844
V5	98.6	101.6	105.3	3900
Valladolid	**99.0**	**102.2**	**102.8**	**3685**
Z1	97.2	101.6	105.4	3138
Z2	101.8	110.7	113.5	3021
Zaragoza	**97.6**	**103.3**	**107.5**	**3109**
Total	**98.9**	**102.8**	**103.5**	

Across years, average productivity was less variable, always in the medium productivity range, from a minimum of 3200 (2005) to a maximum of 4890 kg ha^{-1} (2007). Productivity was higher in the last two years, in which it surpassed 4000 kg ha^{-1}.

To estimate the progress due to selection, we needed to combine the results of years and locations, even though they had different productivity levels. For this purpose we used the relative yield, because it does not fluctuate across years and locations. Rather, it presents values always around 100, and so the values for all trials can be easily combined, although sacrificing the overall productivity perspective.

The averages, for each generation, at each main location and province were calculated in two separate analyses (one for locations, one for province, Table 3.3). Some of the locations were used only occasionally (L4, V2, V4 and Z3). Their inclusion in the analyses increased largely the unbalancedness of the data, therefore affecting the quality of any estimates derived from them. These minor locations were removed from most analyses to reduce the overall unbalancedness, and get better estimates of the factors studied for the main testing locations (Table 3.3).

The comparison of the relative yields at the 10 main locations (during 11 years) indicated that there was progress at most locations over the three generations (Table

3.3). Overall, progress was evident. The means for the three advanced generations were different, F8 presenting the lowest mean and F10 the highest one (Table 3.3). At F8, the overall grain yield was already close to the level of the checks (98.9), and by F10 the outstanding lines clearly surpassed the checks by 3.5%.

Looking at the results of the provinces, in general, progress from F8 to F10 was observed at all four provinces, meaning that the program was successful overall. Differences among provinces were also apparent. The overall progress was larger at Zaragoza and Albacete, and smaller at Lleida and Valladolid.

Progress also differed at the single location level. In F8, only three of the ten main locations reached the yield level of the checks, whereas in F10 these figures were reversed. At F9, the progress was even more evident, as the lines surpassed the checks in all but one location. The highest progress was observed in Z2, where F10 lines surpassed the checks by 13.5%. The progress was large and consistent at the two Zaragoza locations, and smaller at the Lleida locations. In three locations, A1, V1, and L3 the average F10 lines did not reach 100, *i.e.*, their average did not surpass the checks'.

The selection differential (S), genetic gain (G), and realized heritability (H) were calculated for the two selection steps available: F8-F9 and F9-F10.The calculations of S, G and H, were done for sets of lines that were tested in the same location in consecutive years (Table 3.4). The figures indicate an excellent realized heritability was attained for the F8-F9 step, whereas it was low for the F9-F10 step.

Table 3.4. Selection differential (S), genetic gain (G), and realized heritability (H, expressed as percentage of expected gain) calculated for groups of lines in two sets of consecutive generations (F8-F9 and F9-F10) tested in the same locations.

	1st generation		**2nd generation**	**S**	**G**	**H**
	all lines	**selected lines**				
F8-F9	95.9	102.1	102.0	6.24	6.09	97.6
F9-F10	99.9	106.1	100.2	6.28	0.37	5.9

The evaluation of a breeding program that includes testing in multi-environment trials must take into account which are the factors that cause genotypic variation. The

relative size of these components will allow an assessment of the appropriateness of the testing strategies.

The components of variance were calculated for two subsets of data (Table 3.5), made of the sets of years that presented several common checks (Table 3.2).The component of variance for the error was calculated at each individual trial analysis, for each generation at each year and each location. These analyses are routinely done in the Spanish National Barley Breeding Program. The original data for all replicates was not always kept, but the original analyses of variance for most of them are still available. So, the error component of variance was calculated as an average of the error term corresponding to individual trials, weighted according to the degrees of freedom of each individual analysis.

Table 3.5. Components of variance for grain yield in the Spanish Barley Breeding Program. The two periods (1996-2004 and 2005-2008) were chosen according to the presence of sets of common checks.

Random term	1998-2004	2005-2008	Weighted average
n (units)	2172	1865	
Year (Y)	0	1657120	765551
Location (L)	1073410	1158223	1112592
Y × L	2333147	1960767	2161116
Genotype (G)	69426	58736	64487
Y × G	95698	26570	63762
L × G	145824	34329	94316
G × L × Y	295777	361766	326262
Error	208858	235394	224711
Broad-sense h^2	0.70	0.75	0.71

After calculating the components of variance for the two groups independently, a weighted average was calculated for the components of these groups, relative to the number of units which were used in each analysis. This weighted average was assumed to represent the best estimate of the components of variance for the entire dataset under study.

The environmental components of variance were large. ‘Location’ was rather large, and ‘Year’ was highly variable. But, overall, ‘Year × Location’ was the dominant environmental component, which meant that the productivity of locations varied largely between years (Table 3.5).

The calculations of broad-sense heritability in the two analyses were 0.70 and 0.75 respectively, with a general average of 0.71 over the two analyses. These values suggest the possibility to perform selection effectively, though the response may be low some years due to a relatively low genotypic variance (Table 3.5).

An important variance due to 'Genotype' was present in the two analyses. The variance of the GL was larger than that of the GY in the two analyses. This suggests that GEI shows some geographic trend. But the three way interaction (GLY) was larger or even much larger in each analysis, meaning that the geographic trends vary from year to year and are, therefore, unpredictable.

The GEI was broken down into 'Genotype × Province' and 'Genotype within Provinces' interaction for the two balanced series of genotypes and environments. The analyses of variance for these groups are shown in Table 3.6. In most of the groups the variance of 'Genotype × Province' and the 'Genotype within Provinces' terms were rather similar, and in 9 out of 10 of the groups the variance of 'Genotype × Province' (tested against the residual GEI, *i.e.*, the 'Genotype within Provinces' term) was not significant. This means that, actually, the provinces did not explain much of the GEI.

Table 3.6. Summary of the genotype-by-environment interaction factor for ten different analyses of variance for relative yield. The analyses were performed for ten sets of genotypes, which were balanced over two-year trials, either F8 and F9 or F9 and F10.

Years	Generations	Mean squares	
		Genotype × Province	Genotype within Province
1998-1999	F8 - F9	253 ^{ns}	160
1998-1999	F9 - F10	126 ^{ns}	234
2001-2002	F8 - F9	91 ^{ns}	119
2001-2002	F9 - F10	224 ^{ns}	141
2003-2004	F8 - F9	182 ^{ns}	149
2003-2004	F9 - F10	201 ^{ns}	190
2005-2006	F8 - F9	95 ^{ns}	86
2005-2006	F9 - F10	87 ^{ns}	111
2007-2008	F8 - F9	102 ^{ns}	85
2007-2008	F9 - F10	125 *	69

Flowering time data were recorded at most of the locations and years. When flowering date was recorded for a given location, it was done for all trials in that location. The averages of flowering dates for the three generations at all locations were calculated with a mixed model using REML, considering 'generation' and 'location' as fixed factors, and 'year' and its interactions as random factors (Table 3.7).

Table 3.7. Summary of number of lines, flowering date means, minimum, maximum, expressed as the number of days from January 1st", and range of flowering dates for the breeding lines under study (checks excluded), by location and province. Means are REML estimates, whereas minimum, maximum and ranges were calculated with raw values. Averages across provinces and overall average in bold type.

	Lines	Mean	Minimum	Maximum	Range
A1	103	118.3	101	129	28
A2	101	121.7	105	140	35
Albacete	**121**	**120.5**	**101**	**140**	**39**
L1	119	114.1	96	127	31
L2	77	104.8	93	120	27
L3	99	106.3	89	119	30
Lleida	**177**	**106.8**	**89**	**127**	**38**
V1	93	126.2	110	142	32
V3	23	126.7	120	135	15
V5	121	120.0	108	135	27
Valladolid	**135**	**123.3**	**108**	**142**	**34**
Z1	159	120.4	108	141	33
Z2	69	114.1	96	130	34
Zaragoza	**159**	**115.9**	**96**	**141**	**45**
Total		**117.3**	**102.6**	**131.8**	**29.2**

Lleida presented the earliest flowering dates, whereas the latest one was Valladolid. Zaragoza and Valladolid showed the widest flowering time ranges (Table 3.7). The flowering date means were almost constant across locations and provinces for the three generations F8, F9 and F10. The range of flowering dates became narrower with increasing generations, but this could be an effect of sample size.

The regression analysis between grain yield and flowering date was used to further analyze the possible presence of trends in the data. The regression coefficient was calculated using the relative yield and flowering time data of the genotypes under study (lines and checks). The regression coefficient was calculated for all trials run at each year-location combination (usually F8, F9 and F10, taking advantage of the fact

that all three trials were commonly sown on the same date). The regression coefficients between relative grain yield and flowering time were low (Table 3.8). Even though it was statistically significant in some trials, due to the large number of points, the slope of the regression line was almost flat. In some trials (16, *i.e.* about one third), there was a significant negative relationship between relative grain yield and flowering time.

Table 3.8. Results of the regression analyses between relative yield and flowering time in the trials during the period of the study.

Location	Year	Generation	b	R^2	Constant	F pr.	
A1	2003	F8-F10	-0.81	0.039	191	0.093	
A1	2004	F8-F10	-0.30	0.009	135	0.427	
A1	2005	F8-F10	-2.32	0.187	376	<.001	**
A1	2006	F8-F10	-1.30	0.129	248	0.002	**
A1	2007	F8-F10	-2.44	0.106	412	0.006	**
A2	2003	F8-F10	-1.87	0.059	351	0.038	*
A2	2004	F9-F10	0.68	0.018	27	0.440	
A2	2005	F8-F10	-0.12	0.002	112	0.748	
A2	2006	F8-F10	-0.26	0.005	128	0.564	
A2	2007	F8-F10	-3.32	0.127	523	0.002	**
A2	2008	F8-F10	0.34	0.006	56	0.536	
L1	2003	F8-F10	-2.20	0.119	358	0.003	**
L1	2007	F8-F10	1.16	0.163	-25	<.001	**
L1	2008	F8-F10	0.89	0.075	-7	0.022	
L2	1999	F8-F10	-1.63	0.187	270	<.001	**
L2	2002	F8-F10	0.15	0.003	86	0.694	
L2	2004	F8-F10	-1.46	0.052	272	0.053	
L2	2006	F8-F10	-2.09	0.287	306	<.001	**
L3	1998	F8-F10	-1.00	0.030	209	0.135	
L3	2000	F8-F10	0.33	0.010	57	0.517	
L3	2001	F8-F10	0.16	0.002	87	0.746	
L3	2005	F8-F10	0.13	0.001	80	0.781	
L3	2007	F8-F10	0.08	0.002	89	0.709	

*, **, significant at $p \leq 0.05$ and $p \leq 0.01$ respectively

Table 3.8. (continued)

Location	Year	Generation	b	R^2	Constant	F pr.	
V1	1998	F8-F9	-0.32	0.016	143	0.373	
V1	2002	F8-F10	-0.10	0.001	111	0.788	
V1	2005	F9-F10	-2.82	0.417	440	<.001	**
V1	2006	F8-F10	-0.69	0.016	195	0.312	
V1	2007	F9-F10	-1.24	0.134	258	0.043	*
V1	2008	F8-F10	-0.26	0.013	129	0.345	
V3	1999	F8-F10	-0.80	0.065	199	0.056	
V4	1998	F8-F9	-0.77	0.060	219	0.079	
V5	1999	F8-F10	-0.23	0.039	125	0.142	
V5	2000	F8-F10	-1.56	0.365	296	<.001	**
V5	2002	F8-F10	0.14	0.004	82	0.619	
V5	2005	F8-F10	-2.47	0.146	397	0.003	**
V5	2006	F8-F10	-3.30	0.321	485	<.001	**
V5	2007	F8-F10	-0.18	0.006	118	0.532	
V5	2008	F9-F10	1.82	0.187	-110	<.001	**
Z1	2002	F8-F10	0.56	0.054	35	0.074	
Z1	2003	F8-F10	-2.48	0.200	395	<.001	**
Z1	2004	F8-F10	-1.30	0.222	274	<.001	**
Z1	2005	F8-F10	-0.02	0.000	98	0.919	
Z1	2006	F8-F10	-0.55	0.059	159	0.041	*
Z1	2007	F8-F10	-0.85	0.042	202	0.087	
Z2	2001	F8-F10	-0.12	0.002	116	0.775	
Z2	2003	F8-F10	0.25	0.001	82	0.750	
Z2	2004	F8-F10	-0.45	0.006	174	0.539	
Z2	2007	F8-F10	-0.46	0.037	155	0.107	

*, **, significant at $p \leq 0.05$ and $p \leq 0.01$ respectively

3.4. Discussion

The progress associated with selection, the relationship between flowering date and grain yield, and the existence of GEI have not been studied previously in the Spanish National Barley Breeding Program. The success of the program is evident, based on its capacity to produce improved cultivars, which are being readily adopted by the industry and the producers. Nevertheless, a systematic retrospective analysis may offer clues about the effectiveness of the practices used, and help to identify possible weaknesses of the program.

It is assumed that each set of checks marked, at each year and location, the threshold of agronomic excellence for the program. Therefore, the overall relative yield means (Table 3.3) indicate a significant progress in the barley breeding program over the period studied. The difference between all three generations was remarkable, and in the end surpassed the yield of the checks. It seems that the overall progress slowed down after F9, however, as there was an increase of only 0.7% from F9 to F10 compared to 3.9% from F8 to F9. This may have been affected by the lower selection pressure applied from F9 to F10 (Table 3.2).

Another conclusion from the overall means is that the program already achieved a good productivity level at F8, with a mean performance quite close to the checks (98.9%). A similar trend in the performance of selected lines and check cultivars has been reported by Khalil et al. (2004) in a wheat breeding program. This may be the result of an efficient selection over the generations up to F8 or, alternatively, could mean that the productivity level achieved for the materials in the program is high from the very beginning. It is not inferred from the data which of these hypotheses is more likely. But the fact that most of the parents currently used in the program are recycled advanced lines suggests that the program may be reaching a mature stage, in which productivity level is optimized across all generations.

The true gain attained in the program is probably higher than the calculated for the relative yields. As the checks were gradually replaced over the years, it can be safely assumed that the yield level of the checks also rose over the years, as the new checks replaced older cultivars that became obsolete. In consequence, the gain calculated for relative yield is most likely an underestimation of the true gain in kilograms per hectare.

At the province level, there was higher progress in Albacete and Zaragoza, compared to Lleida and Valladolid. The small progress in Lleida and Valladolid may have been partially caused because, at these provinces, the F8 already showed a very

high grain yield level, and subsequent progress could have been more difficult to attain. Though the gain in Albacete was apparent, the final yield level at F10 barely reached the level of the checks, whereas at the other three provinces, F10 lines level clearly exceeded the checks.

Gain from selection was apparent at most locations. In three locations, F10 relative yield was below 100, *i.e*, the program was less effective in finding superior cultivars for these locations. The case of V1 was not surprising, as it was actually a conglomerate of different locations close to Valladolid city and, in consequence, a larger effect of GEI (lowering genetic gains) is expected. On the other hand, the case of A1 (Albacete dry-land) is worrying, as it seems that the program is not achieving its objective at the lowest yielding location. The low progress at this location affected the result of Albacete as a whole, and explains the unsatisfactory overall results at this province. It can be speculated that the program is not addressing properly the adaptation to the poorest growing conditions. To test this, we calculated a correlation coefficient between the program progress (the difference between F8 and F10) and the mean grain yield at the 10 main locations. The *r* value was just -0.12, indicating that the relationship between response to selection and productivity level was probably negligible. Finally, there is no plausible explanation for the low progress at L3.

Positive genetic gains from F8 to F9 were found (as in the studies of Khalil et al. 2004, 2010). But it was very low, almost negligible, from F9 to F10, though this was affected by other factor that will be discussed below. In any case, this indicates a lower effect of selection after F9. There were some lines tested for more than one year in F10. These lines used to be the best lines of the trial, that were maintained in the program for some additional years before taking the final decision of releasing them as cultivars or recycling them as parents. This was the reason of the apparently different results for the F10 in Table 3.3. In Table 3.4, the results of only the first year of F10 evaluation are presented. Actually, the lines that were kept in the program for additional years at the F10 had a relative yield above 105 in the second and third years of evaluation. Their absence in the calculations of realized heritability swayed the overall F10 average slightly downwards. The reasons for not reaching a realized heritability of 1 are the presence of error and of GEI.

Regarding components of variance, 'Year' variance was very different between the two analyses done (Table 3.5). This is explained by the rather constant yearly averages observed during the first period analyzed (1998-2004), compared to the highly

variable averages observed in the second period (2005-2008, Table 3.5). This was not unexpected, as large yearly fluctuations are common in Mediterranean environments (Turner 2004). Genotypic variance was detected in the two analyses performed, meaning that there were true genotypic differences still at this stage of the program. It had comparable size to the GL and GY interactions. In a similar study focused on a wheat breeding program, Roozeboom et al. (2008) found a genotypic variance almost twice as large as the GL and GY variances. Similar figures were found by Thomason and Phillips (2006), for wheat breeding in Virginia. Their studies are relevant to ours because they were also testing advanced materials (candidate cultivars) in large geographical areas with highly variable environments (especially Roozeboom et al. 2008). This shows that the situation for the Spanish barley breeding program presents even higher challenges, as the interactions involving the 'Genotype' factor were higher.

GL in the data was rather high, indicating the presence of a geographical factor in the GEI. When this happens, the breeders are confronted with the issue of whether the program should target wide adaptation, or it should be split between different locations due to the high GL interaction. But the results in the two analyses comprising the entire 11 years (Table 3.5) indicate that the 3-way interaction, between genotypes, locations and years was the principal source of variance. Therefore, the geographical patterns varied between years and were not predictable. Hence, a split of the program based on more stable geographic sub-zones is not advisable.

Consistent with this, it is observed that there was almost no Genotype × Province interaction (Table 3.6). Therefore, whatever factors were causing GEI in this dataset, they seemed not related with geographical division at the province level. This finding reassures that the current strategy, combining the results of the four provinces is appropriate. Cullis et al. (2000) found a similar situation when analyzing series of variety tests conducted for several crops in Australia. They found that classical geographic zonation had little meaning under the light of actual variance components calculated for them.

The presence of locations from all provinces ensures a good coverage of all GEI situations possible. In other words, the representativeness of the locations is good. It may be argued that the two Albacete locations (actually, two trials in the same location) are redundant to some extent. But the very distinct results observed in response to selection between A1 and A2 (Table 3.3) suggests that these two trials are probably giving different, non-overlapping information.

The changes in flowering date means and ranges indicate that, even though this trait has undergone several rounds of selection by this stage of the breeding program, there was still a slight selection towards earliness from F8 to F10 (Table 3.7). There was a spread of flowering dates across locations, proportional to the mean temperatures over the growing season, with colder locations (from Valladolid) reaching flowering later than warmer locations (for instance, L2 and L3). A dynamic relationship of flowering date with barley yield in Spanish environments was already found by Cuesta-Marcos et al. (2009). Though some water stress is almost always present in our conditions, timing and intensity of this stress varies widely. Therefore, it is not surprising that the relationship between flowering date and yield changed depending on the environment. The regression coefficients between relative grain yield and flowering time were, in general, rather low (Table 3.8) indicating that the relationship between yield and flowering time overall was weak in the locations under study at this advanced stage of the program. This relationship would possibly be more tight if the selection up to F8 had not removed already the most early and, especially, late genotypes.

In summary, there was progress due to selection over the last generations of the Spanish National Barley Breeding Program. Grain yield increased from F8 to F10, surpassing the level of the checks. We can conclude that the program is reaching its main goal of producing and identifying superior barley genotypes with high yield potential and stability suitable across all Spanish barley growing regions. The effectiveness of selection was satisfactory across all four provinces, though differences were observed among particular locations. It was also more effective up to F9, whereas there was little gain in the last generation.

These results also suggest that it would be unpractical to run separate breeding programs for separate provinces or locations (either considering an entire program or just the last generations). If we had found clear differences in GEI among provinces, the situation might have been different, as provinces are large geographical units, which may justify additional efforts. But the structure of the components of variance and the absence of a stable geographic structure of the GEI, it seems sensible that the program continues with the same geographic structure, using the same provinces and locations.

The definitive proof of the success of a breeding program is the adoption of the varieties released by the industry. Cultivars Cierzo, Estrella and Yuriko, released over the last five years performed very well in independent trials, and are currently under exploitation by three different companies.

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Chapter 4

Relationship between genotype-by-environment interaction and vernalization requirement in barley grown in Spain

4.1. Introduction

Plant breeders aim to obtain genotypes with stable and high performing phenotypes across environments. However, the environment and genotype by environment interaction affect the phenotype of cultivars and breeding lines, especially if the target environments are not homogeneous (Nurminiemi et al. 2002).

Genotype-by-environment interaction (GEI) reflects the various responses of genotypes to environmental conditions. Some genotypes have a stable phenotypic performance in a wide range of environmental conditions, while others display considerable variation across environments. GEI can be statistically defined as the difference between the phenotypic value and the value expected from the mathematical model of observations that takes into account the general mean as well as genotypic and environmental main effects (Warzecha et al. 2011). The decisions about the commercial value of new crop varieties are usually based on data from Multi Environment Trials (METs), conducted over several locations and years, across the target environment (Smith et al. 2001). MET series are designed to cover a range of agro-ecological conditions that may occur in the target environment (Romagosa et al. 2009). The presence of high GEI in METs means that the genotypes tend to react differently to varying environmental conditions (Comadran et al. 2011). These varying conditions may be climatic, edaphic, biotic, or anthropic (if caused by crop management). The specific causes of GEI in barley trials were reviewed by Voltas et al. (2002).

In barley breeding, and in many aspects of barley research, GEI is of primary importance because it often complicates testing and selection of superior genotypes, thus reducing genetic progress in breeding programs (Voltas et al. 2002; Rodriguez et al. 2008). Targeting cultivars to a specific location is difficult when GEI is present, since yield is less predictable and cannot be interpreted based only on genotype and environment means (Ebdon and Gauch 2002). This issue is particularly critical in Mediterranean areas, where barley growth is often hampered by the occurrence of drought and high temperatures, and large inter-annual changes in climate factors (Voltas et al. 1999).

The additive main effects and multiplicative interaction (AMMI) model is a statistical model for describing and understanding GEI (Gauch 1992). It is a hybrid analysis that incorporates both the additive and multiplicative components of the two-way data structure (Kaya et al. 2002). AMMI analysis has been shown to be effective in understanding complex GEI (Tarakanovas and Ruzgas 2006; Balestre et al. 2009). An

effective tool to diagnose GEI patterns graphically using the results of the AMMI analysis is the biplot representation (Thillainathan and Fernandez 2001).

The conditions for production of winter cereals across Spain are variable but, in general, fall under one of the several subclasses of Mediterranean climates. These include mild to cold winters, rapidly increasing temperatures in spring, very high temperatures in summer, and limited rainfall. Producers have long known that not all cultivars are equally suited to each particular region. They usually prefer autumn over winter sowings in order to take advantage of the longer growing period, and of the periods of maximum rain, thus increasing yield potential. Choosing the type of cultivar to grow under these conditions is not easy. Choices range from mid- to late-spring cultivars, with some degree of freezing tolerance, to strict winter cultivars with a strong vernalization requirement and freezing tolerance. The choice of growth habit is made based on the frequency of occurrence of harsh winters, which follows geographic clines. Therefore, a detailed knowledge of the genetic factors affecting barley development in Mediterranean environments is very important to respond to the challenge of developing cultivars suited to each specific situation (Cuesta-Marcos et al. 2008).

Barley cultivars with “winter” growth habit need a period of low temperature to satisfy the vernalization requirement. Fulfillment of this requirement promotes flowering, which is also promoted by long days (once vernalization is satisfied). “Spring" barley does not require vernalization, and is usually insensitive to photoperiod (von Zitzewitz et al. 2005). Finally, the “facultative” growth habit is typically utilized to refer to genotypes that are as tolerant of low temperatures as winter varieties, but lack a vernalization requirement (von Zitzewitz et al. 2005).

The objectives of this study were to i) measure GEI for barley across representative barley growing regions of Spain based on the locations used in the Spanish National Barley Breeding Program, ii) explore if this interaction is affected by the growth habit of the cultivars, and iii) determine if GEI is related to climatic variation among the trials.

4.2. Materials and Methods

This study used data from the Spanish National Barley Breeding Program, which is described in detail in Gracia et al. (2012). The trials of the Spanish National Barley Breeding Program are carried out by four public research organizations in four provinces: Albacete (Instituto Técnico Agronómico Provincial, ITAP), Lleida (Instituto de Investigación y Tecnología Agroalimentarias, IRTA), Valladolid (Instituto Tecnológico Agrario de Castilla y León, ITACyL) and Zaragoza (Estación Experimental de Aula Dei, EEAD-CSIC) (Table 4.1). In each province, several locations are used for testing. However, not all locations are used every year. The trials are rotated between locations in Valladolid and Lleida, whereas locations in Albacete and Zaragoza are always the same. Usually, advanced yield trials are carried out in at least two locations per province each year, although data from some locations are usually discarded due to a variety of reasons (crop failures, sowing errors, bad quality of data, etc). All locations are non-irrigated, except Gimenells (L3), where irrigation is provided when drought is severe, and Albacete-irrigated (A2), which receives irrigation regularly. In the province of Valladolid, one of four experimental farms near the capital city is used in different years. These farms are close enough to each other to be considered as a single location (V1).

The data used for this study were generated in 68 environments (year-location combinations), in which 183 advanced trials were grown during 11 seasons from 2000 to 2010 (the year denotes harvest year), at up to 11 locations (3-10 per year), and up to three trials per year for the advanced generations (Table 4.1). At some locations, some trials were lost or not sown for a variety of reasons.

The purpose of these trials was to test the advanced breeding lines against a set of four check cultivars. Experimental lines in the trials belong to three generations: F8, F9, and F10. Each of the three generations was grown in a separate, but adjacent, trial. An alpha-lattice design with three replications was used for each trial. Each plot occupied 7.2 m^2 (6 × 1.2m), with either 6 or 8 rows. Each trial included four checks, which are the benchmark against which candidate lines are compared. These checks are among the best cultivars grown in Spain at each time. The same checks were used for each of the three generations, so each check was actually replicated up to nine times per environment.

Table 4.1. Summary of the trials under study during 2000 to 2010.

Province	Location	Code	00	01	02	03	04	05	06	07	08	09	10
Albacete	Albacete dry-land	A1				x	x	x	x	x		x	x
	Albacete irrigated	A2				x	x	x	x	x	x	x	x
Lleida	Artesa	L1			x	x			x	x	x	x	x
	Bell-lloc	L2			x		x		x		x		x
	Gimenells	L3	x	x		x		x		x		x	
	Solsona	L4						x		x	x		
Valladolid	Valladolid capital	V1			x			x	x	x	x	x	x
	Villahoz	V2								x			
	Ceinos	V3	x										
	Macotera	V5	x		x	x		x	x	x	x	x	
Zaragoza	Sádaba	Z1	x	x	x	x	x	x	x	x			x
	Vedado	Z2		x		x	x			x		x	x

In this work, the genotypes studied were divided in two sets: first, the checks of the trials, which constitute sets of common genotypes tested over a prolonged period of time. These well-known checks were used to describe the general patterns of GEI. According to their growth habit, the checks used were winter, spring or facultative cultivars (Table 4.2). This is not unusual for Spain, as facultative and even spring cultivars may be sown in the autumn over large areas of the country. A total of six checks were evaluated during the time frame sampled in this study. Some checks changed over time, replaced by new, more successful cultivars. The dataset was, therefore, divided in two periods, based on the checks used. In period 1 (5 seasons, 2000 to 2004) the checks were Alpha, Barberousse, Graphic and Zaida. In period 2 (6 seasons, 2005 to 2010) the checks were Barberousse, Cierzo, Graphic and Hispanic.

Table 4.2. Summary of the check cultivars in advanced trials (F8, F9, F10) of the Spanish Barley Breeding Program during 2000 to 2010.

Genotype	Growth habit	Row type	Period 1 (2000-2004)	Period 2 (2005-2010)
Alpha	Winter	Two	x	
Barberousse	Winter	Six	x	x
Cierzo	Intermediate	Six		x
Graphic	Spring	Two	x	x
Hispanic	Winter	Two		x
Zaida	Spring	Two	x	

The second set of genotypes comprises part of the lines under evaluation in the same advanced trials of the breeding program. The checks and the breeding lines

reaching F8 are routinely characterized with a panel of markers, including *VrnH1* and *VrnH2*, the main vernalization genes in barley, and responsible for an important proportion of GEI in our conditions (chapter 5). This allows the discrimination of several major classes of genotypes, according to the expected growth habit (mostly based on vernalization response), mainly 'winter', 'spring', and 'intermediate'. The subset of lines that will be analysed here comprises only the genotypes which had either of two specific combinations of alleles at *VrnH1* and *VrnH2*. The two classes of gnotypes tested were the most abundant in the program. Although other type of lines (spring, for instance) were tested, their frequencies were too low to derive any conclusions.

The phenotypic traits evaluated were grain yield, expressed in kg per hectare at 10% moisture, and days to heading, recorded as the date when at least 2 cm of the awns were visible in 50% of the tillers of each plot (developmental stage 49 in the Zadoks scale, Zadoks et al., 1974). Days to heading was expressed as the number of days after January 1st. Relative grain yield for each genotype was estimated as a percentage compared with the average grain yield of the checks at each particular trial, to homogenize the results among years and locations.

Minimum, average and maximum monthly temperature and rainfall data were collected from the meteorological stations nearest to the locations under study (Table 4.3). Climatic data were collected for all trials except 00V3, 05L4, 07L4, 07V2 and 08L4 (complete data in Annexes 4.1 and 4.2).

Table 4.3. Coordinates of the testing locations of the National Barley Breeding Program and nearby meteorological stations used to collect long term climatic data.

Location	Latitude	Longitude	Meteorological station	Latitude	Longitude
A1 - A2	38°59′N	1°51′W	Albacete,OBS.	39°0′N	1°51′W
L1	41°33′N	0°42′E	Artesa de Segre	41°53′N	1°02′E
L2	41°37′N	0°46′E	Lleida	41°37′N	0°37′E
L3	41°39′N	0°23′E	Lleida	41°37′N	0°37′E
V1	41°43′N	5°32′O	Valladolid	41°38′N	4°43′W
V5	40°49′N	5°17′W	Villar de Gallimazo (Pedrezuela S. Bricio)	40°58′N	5°18′W
Z1	42°17′N	1°16′W	Sadaba	42°170′N	1°16′W
Z2	41°51′N	0°39′W	Zuera Aspasa	41°52′N	0°45′W

The data set is unbalanced because it was collected over 11 years, and the trials were not carried out at exactly the same locations every year (Table 4.1). Least square

(LS) means of yield and days to heading were calculated for each check at each trial using a mixed model approach (REML), implemented in the software package Genstat 14 (VSN International 2011). The trials and genotypes were considered fixed factors and generations were considered as a random factor (similar, in this case, to replicates). For days to heading in period 2, the factor generations was omitted from the analysis because its effect was negligible. The least square means resulting from these analyses were used to construct AMMI models and biplots for the two periods, using Genstat 14 (VSN International 2011). Correlation coefficients between the first two significant principal components of the AMMI analysis and the climatic variables were also calculated, using the appropriate routines of Genstat 14.

For the analysis of the dataset of the checks, the means of squares and sums of squares for grain yield and days to heading for genotype, environment and genotype by environment interaction, were derived from the output of the REML analysis. These analyses were combined with the AMMI analysis performed on the least square means and, to account for the loss of the replicates (generations) in this analysis, the sums of squares for PCA1 and PCA2 were multiplied by the actual average of replicates per environment, which was a number between 2.6 and 2.9, because in some cases there were less than 3 trials per environment.

Seasonal values were calculated for the climatic variables, averages for the temperatures, and cumulative values for precipitation (Annexes 4.1 and 4.2). "Winter" values were calculated with monthly values for January, February and March; "spring" values were calculated with the months of April, May and June. For further analyses, the trials were divided in three temperature classes, according to their average winter temperatures: "low temperatures", from 3.7º to 5.7ºC, "intermediate temperatures" from 5.8º to 7.7ºC, and "high temperatures" from 7.8º to 9.8ºC. A REML analysis was done for the variables relative grain yield and days to heading, with genotypic classes according to the *VrnH1* alleles and temperature classes as sources of variation. From this analysis, the averages of the lines carrying the *VrnH1* allele like cultivars Orria or Cierzo (*VrnH1-4*), and the recessive *vrnH1* winter allele across the three classes of temperatures were calculated and compared.

4.3. Results

Patterns of GEI in check cultivars

Grain yield varied remarkably across environments (Table 4.4). Each genotype also showed a wide yield range across environments. In period 1, Graphic was the best check in 8 environments, followed by Barberousse, in 4 environments (Annex 4.3). In the second period, the two new checks were clearly superior to the old ones. In this period, Cierzo was the best in 24 environments, followed by Hispanic (8), and Graphic (7). In this second period, Graphic seemed to have an advantage in the highest yielding trials (Annex 4.4).

Table 4.4. Mean, minimum and maximum of the productivity average of grain yield and days to heading for the checks and test lines studied at multienvironment trials, in the Spanish Barley Breeding Program during the period 2000 to2010.

		Grain Yield (kg ha^{-1})			**DHE** (days from January 1st)		
Period	Genotype	Trials (n)	Min-Max	Mean	Trials (n)	Min-Max	Mean
				Checks			
2000-04	Alpha	24	1723-8868	5036	19	100-134	116
	Barberousse	24	1661-10043	5200	19	100-133	116
	Graphic	24	1859-9841	5373	19	100-141	118
	Zaida	24	1920-8893	4751	19	96-135	115
2005-10	Barberousse	44	1284-10180	5698	39	100-150	118
	Cierzo	44	1314-11105	6183	39	101-150	119
	Graphic	44	1284-11876	5836	39	96-140	120
	Hispanic	44	1432-10569	5769	39	95-137	115
				Test lines			
2000-10	*VrnH1-4*	68	790-12567	5472	57	95-142	118
	vrnH1(winter)	70	1154-11020	5696	58	94-138	117

In the AMMI analysis for yield, the first principal component of the GEI captured 49.2 and 46.1% of the GEI sum of squares of grain yield for the two periods, respectively. The second principal component explained 29.6 and 28.9% of the GEI sum of squares of grain yield (Table 4.5).

Table 4.5. Analysis of variance and AMMI for grain yield and days to heading of the genotypes across environment during the two time periods, 1 and 2. Colours indicate two different partitions of the GEI term.

		Grain yield			Days to heading		
	Source	df	MS		df	MS	
Period 1	Genotype	3	4422175	**	3	73.7	**
	Environment	23	39356710	**	18	987.4	**
	GEI	69	548721	**	54	15.7	**
	IPCA 1	25	743450	**	20	30.8	**
	IPCA 2	23	486679	*	18	7.5	
	GEI residual	21	384852		16	6.0	
	G.Temp_class	6	1154774	**	6	15.2	**
	G. within Temp_class	63	491002	*	48	15.8	**
	Residual	154	216032		122	5.2	
Period 2	Genotype	3	5560748	**	3	503.7	**
	Environment	43	49408567	**	38	659.1	**
	GEI	129	659855	**	114	12.9	**
	IPCA 1	45	871317	*	40	22.1	**
	IPCA 2	43	572130	*	38	9.6	
	GEI residual	41	519767	*	36	6.3	
	G.Temp_class	6	1592661	**	6	12.5	**
	G.within Temp_class	123	614352	*	108	12.9	**
	Residual	302	309791		273	7.7	

The AMMI biplots for grain yield were generated using genotypic and environmental scores of the first two AMMI components, (Fig. 4.1). The biplot has four sections, depending upon signs of the genotypic and environmental scores. In period 1, the most noticeable feature was that the four cultivars were well spread in the graph, over three quadrants, indicating different genotypic reactions specific to each one of them. The first component placed the two winter cultivars on the positive side, whereas Graphic, a spring cultivar had a large negative loading. The other spring cultivar, Zaida, had a negligible score on the first axis, most likely because it was the worst cultivar, in general, and its interaction with the environment was the lowest of all. The same occurred in the biplot of period 2, in which the four cultivars were placed each in one quadrant of the plot. In this case, however, it was the second component that seemed to divide the genotypes according to growth habit, opposing winter cultivars 'Barberousse and Hispanic' to the spring cultivar Graphic, with intermediate cultivar Cierzo at an intermediate position, not far from the horizontal axis (Fig. 4.1).

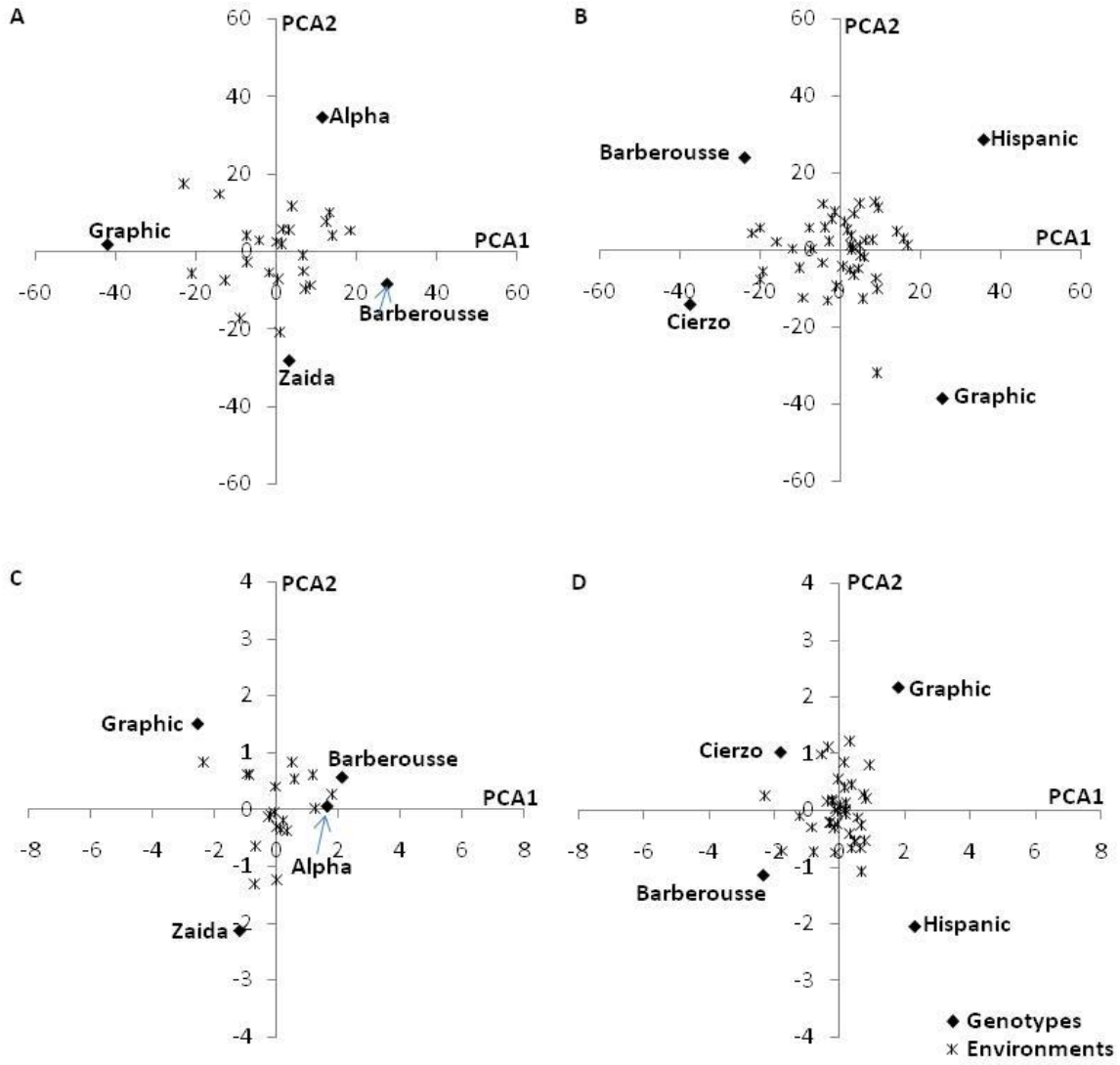

Figure 4.1. AMMI 2 model biplots for grain yield and days to heading of the winter and spring check cultivars during periods 1 (A grain yield, C days to heading) and 2 (B grain yield, D days to heading).

To verify if the principal components could be related with responses to climatic conditions, we calculated linear correlation coefficients between the environmental scores of the principal components and a series of climatic variables (mean, minimum and maximum monthly temperatures, seasonal temperature and precipitation). A clear pattern emerged when looking at the correlations with temperature. The first principal component of period 1 and the second principal component of period 2 were significantly correlated with winter temperatures (Table 4.6).

Table 4.6. Correlation coefficients between the first two principal components of the AMMI analyses for grain yield and the minimum, average, maximum monthly temperature and variables expressed as seasonal averages of temperatures.

		Period 1		Period 2	
		IPCA1	IPCA2	IPCA1	IPCA2
Minimum temperature	Nov	0.04 ns	0.17 ns	0.08 ns	**-0.33** *
	Dec	0.04 ns	-0.12 ns	0.07 ns	0.22 ns
	Jan	-0.13 ns	0.24 ns	**-0.40** *	-0.22 ns
	Feb	**-0.73** **	0.07 ns	-0.22 ns	**-0.37** *
	Mar	**-0.55** **	-0.08 ns	0.15 ns	-0.17 ns
	Apr	-0.33 ns	0.05 ns	-0.13 ns	-0.27 ns
	May	**-0.44** *	-0.08 ns	0.01 ns	-0.18 ns
	Jun	-0.10 ns	0.05 ns	0.06 ns	-0.04 ns
Average temperature	Nov	0.00 ns	0.12 ns	-0.03 ns	**-0.38** *
	Dec	0.04 ns	-0.19 ns	0.08 ns	0.08 ns
	Jan	-0.13 ns	0.21 ns	-0.15 ns	**-0.41** **
	Feb	**-0.66** **	-0.02 ns	-0.22 ns	**-0.48** **
	Mar	**-0.57** **	-0.18 ns	0.12 ns	-0.17 ns
	Apr	-0.39 ns	-0.04 ns	-0.13 ns	-0.24 ns
	May	-0.40 ns	-0.13 ns	0.12 ns	-0.17 ns
	Jun	-0.06 ns	0.003 ns	0.14 ns	-0.05 ns
Maximum temperature	Nov	-0.03 ns	0.05 ns	-0.13 ns	**-0.37** *
	Dec	0.03 ns	-0.26 ns	0.08 ns	-0.04 ns
	Jan	-0.12 ns	0.16 ns	0.07 ns	**-0.40** *
	Feb	**-0.58** **	-0.06 ns	-0.22 ns	**-0.52** **
	Mar	**-0.50** *	-0.25 ns	0.06 ns	-0.14 ns
	Apr	-0.40 ns	-0.10 ns	-0.14 ns	-0.21 ns
	May	-0.34 ns	-0.15 ns	0.18 ns	-0.15 ns
	Jun	-0.01 ns	-0.04 ns	0.22 ns	-0.05 ns
Winter[1], minimum temperature		-0.52 *	0.10 ns	-0.19 ns	-0.34 *
Winter, average temperature		-0.61 **	0.005 ns	-0.12 ns	-0.44 **
Winter, maximum temperature		-0.59 **	-0.07 ns	-0.05 ns	-0.45 **
Spring[1], minimum temperature		-0.31 ns	0.01 ns	-0.01 ns	-0.17 ns
Spring, average temperature		-0.31 ns	-0.06 ns	0.06 ns	-0.16 ns
Spring, maximum temperature		-0.29 ns	-0.11 ns	0.11 ns	-0.15 ns

[1] Winter: January, February and March; Spring: April, May and June.

The other components showed negligible correlation coefficients with the temperature variables. Only one was significant, January minimum temperature with PCA1 for period 2 (Table 4.6), but the correlations with the seasonal averages of the climatic variable were very low. The correlations with precipitation were only apparent for the second principal component of period 2 for the months of December, March and April (Table 4.7) but, again, the correlations with the seasonal averages were very low for both components in the two periods (Table 4.7).

Table 4.7. Correlation coefficients between the first two principal components of the AMMI analyses for grain yield and rainfall.

		Period 1		**Period 2**	
		IPCA1	IPCA2	IPCA1	IPCA2
Rainfall	Nov	-0.05 ^{ns}	0.27 ^{ns}	0.27 ^{ns}	-0.01 ^{ns}
	Dec	0.10 ^{ns}	-0.16 ^{ns}	-0.24 ^{ns}	**0.34** *
	Jan	0.12 ^{ns}	-0.06 ^{ns}	-0.36 *	0.13 ^{ns}
	Feb	0.36 ^{ns}	-0.03 ^{ns}	-0.07 ^{ns}	-0.01 ^{ns}
	Mar	0.28 ^{ns}	0.08 ^{ns}	-0.01 ^{ns}	**-0.42** **
	Apr	-0.001 ^{ns}	0.25 ^{ns}	-0.20 ^{ns}	**-0.41** **
	May	0.18 ^{ns}	0.12 ^{ns}	-0.26 ^{ns}	-0.04 ^{ns}
	Jun	-0.42 *	0.08 ^{ns}	-0.11 ^{ns}	-0.01 ^{ns}
Winter[1] Rainfall		0.41 ^{ns}	-0.02 ^{ns}	-0.20 ^{ns}	-0.13 ^{ns}
Spring[1] Rainfall		-0.08 ^{ns}	0.23 ^{ns}	-0.28 ^{ns}	-0.23 ^{ns}

[1] Winter: January, February and March; Spring: April, May and June.

The check genotypes also showed large differences across environments for days to heading (Table 4.4). Days to heading from January 1st ranged from 96 to 141 days in period 1 and from 95 to 150 days in period 2 (Table 4.4). For instance, Graphic ranged between 96 and 139 days in period 1 and 96 to 140 days in period 2. During period 1, the range of flowering dates was smaller than in period 2. The earliest heading dates were quite consistent across cultivars and seasons, just six days, from 95 to 101. The latest heading dates, however, presented higher variation, ranging from 133 to 150. This suggests that the conditions for reaching flowering were variable, surely affected by sowing times, but also due either to a difference in the accumulation of thermal time, or to differences in vernalizing potential at the environments (Annexes 4.5 and 4.6).

The differences between genotypes as well as the GEI were significant, at the two periods (Table 4.5). In the AMMI2 biplot for days to heading, the first principal

component axis (PCA1) in period 1 captured 70% of the GEI sum of squares of days, and 53.7% in period 2. The second principal component axis (PCA2) explained 15.4 and 22.1% of the GEI sum of squares, respectively, in the two periods (Table 4.5).

The biplot for the first two principal components for period 1 divided the genotypes into two sections, one with the winter cultivars 'Alpha and Barberousse' and another one with the spring genotypes, 'Graphic and Zaida, in the same way as the AMMI biplot for grain yield. For period 2, we also observed a similar situation compared with grain yield, with the four genotypes allocated at a different quadrant of the plot (Fig. 4.1). In this case, there was a significant correlation of PCA1 with temperature across the entire season for period 1, which was almost absent from period 2 (Table 4.8).

Table 4.8. Correlation coefficients between the first two principal components from the AMMI of days to heading and the minimum, average, maximum monthly temperature and variables expressed as seasonal averages of temperature and rainfall.

	Period 1				**Period 2**			
	IPCA1		IPCA2		IPCA1		IPCA2	
Winter[1], minimum temperature	0.51	*	-0.10	ns	-0.12	ns	-0.16	ns
Winter, average temperature	0.55	*	-0.12	ns	-0.11	ns	-0.16	ns
Winter, maximum temperature	0.54	*	-0.12	ns	-0.09	ns	-0.13	ns
Spring[1], minimum temperature	0.54	*	-0.26	ns	-0.07	ns	-0.35	*
Spring, average temperature	0.56	*	-0.23	ns	-0.04	ns	-0.29	ns
Spring, maximum temperature	0.57	*	-0.21	ns	-0.02	ns	-0.22	ns
Winter Rainfall	-0.19	ns	-0.52	*	0.06	ns	0.20	ns
Spring Rainfall	-0.15	ns	0.07	ns	0.004	ns	0.42	*

Again, PC1 for period 1, and PCA2 for period 2 seemed related with growth habit. This was confirmed by a strong and significant correlation coefficients of the environmental scores of these components with the difference in days to heading between the winter and spring cultivars at each environment (Barberousse-Alpha vs Graphic-Zaida in period 1, Barberousse-Hispanic vs Graphic in period 2), with coefficients of 0.99 and 0.85, respectively.

Patterns of GEI at selected groups of advanced breeding lines

To look for further confirmation of the relation of GEI for grain yield with temperature, we used the data from advanced breeding lines of the program. Several analyses were performed to confirm if they followed the same trends as observed for the

check cultivars. The advanced lines were characterized according to the alleles they presented at the two main vernalization genes, *VrnH1* and *VrnH2*. It had been observed during the routine checkup of the lines with molecular markers in the breeding program that a majority of the lines selected conformed to either one of two haplotypes. One corresponded to the lines which had the *VrnH1-4* allele derived from cultivar Orria. This cultivar is an "intermediate" variety, with a functional *VrnH2* allele, and a shortened gene at *VrnH1,* which reduces the vernalization requirement compared to the allele in winter cultivars as Barberousse, like the check cultivar Cierzo. The second class were the lines which had the functional *VrnH2* allele and the typical recessive winter allele *vrnH1,* as check cultivars Barberousse, Alpha and Hispanic. In the period studied (2000-2010), a total of 122 lines of these two haplotypes were tested in the program, corresponding to 56 different crosses, and tested over a maximum of three seasons (in F8, F9 and F10, i.e. three seasons at most). All of them had the 'winter' active allele at *VrnH2*, whereas 64 of them had the *VrnH1-4* allele derived from cultivar Orria (section 5.4 of this thesis), and 58 had the typical recessive *vrnH1*winter allele, from a wide variety of parents.

The averages for the sets of lines with each of the haplotypes at *VrnH1*-*VrnH2* tested at each environment were incorporated with the averages of the check cultivars to construct new AMMI biplots for the two periods of study. The results were very similar to the AMMI of the checks. The advanced lines showed the GEI patterns close to their most similar checks (Fig. 4.2). The lines with typical winter *VrnH1* were located closer to the winter check cultivars at the two time periods, particularly regarding the axes already identified as related to winter temperature. The lines like Orria fell in the same section as check cultivar Cierzo (of which Orria is a parent) in period 2. In period 1, in which there was no check cultivar representative of this kind of lines, they were located in a different quadrant than all the checks, and opposite to the winter checks and winter lines according to the first principal component, the one related with growth habit. Therefore, even the highly diverse set of test lines, distributed over the trials in a highly unbalanced way, presented GEI patterns consistent with expectations according to their haplotypes at *VrnH1* and *VrnH2*.

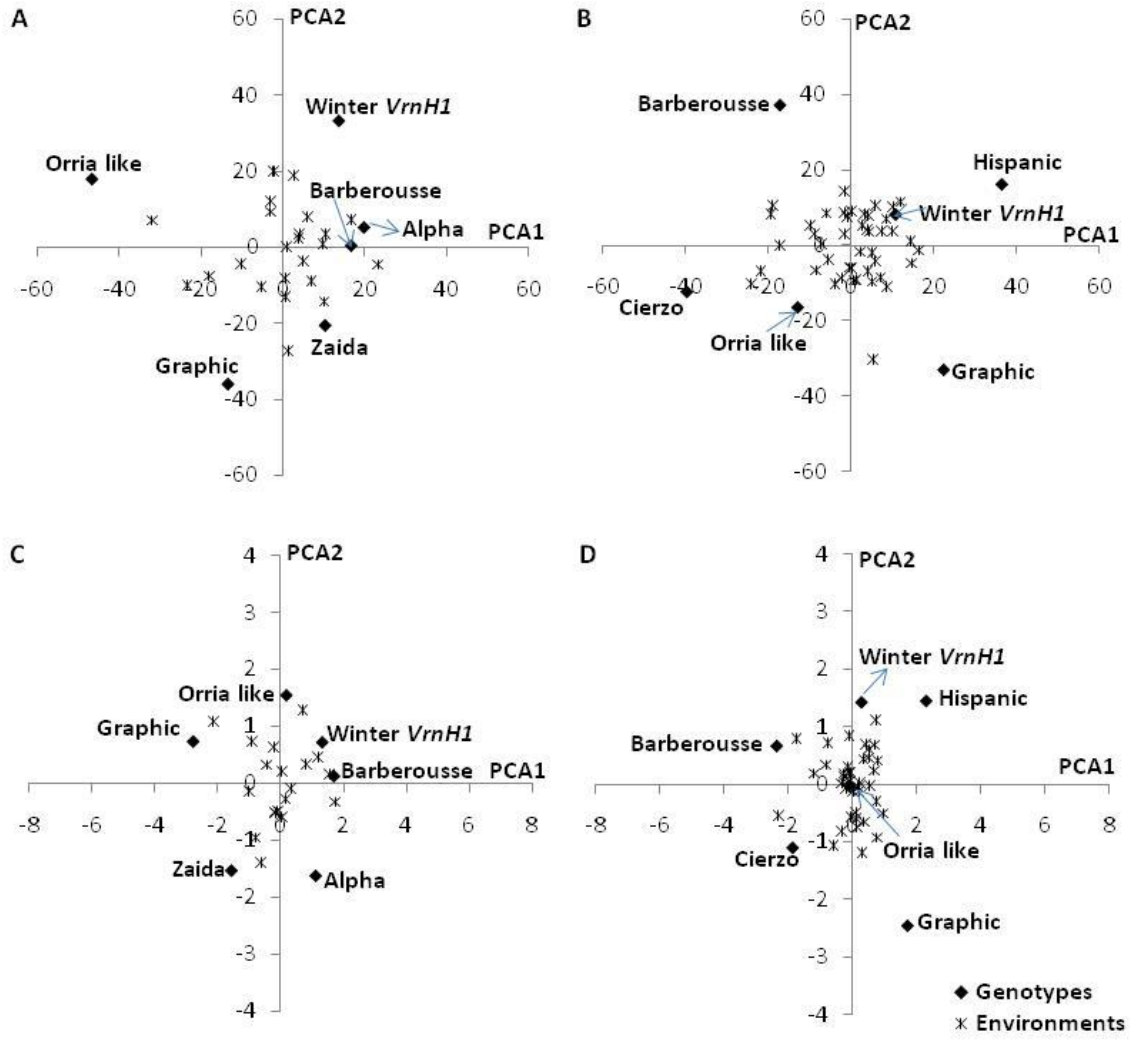

Figure 4.2. AMMI-2 model biplots for grain yield and days to heading of the check cultivars and the averages of the advanced lines (divided by the allele present at *VrnH1*, *VrnH1-4* or *vrnH1*) during periods 1 (A grain yield, C days to heading) and 2 (B grain yield, D days to heading).

Relationship of GEI for grain yield of barley with winter temperature

The relationship of GEI with winter temperature was further examined attending at the performence of check cultivars and advanced lines at the environments divided in three classes, according to the average winter temperatures, as explained in section 4.2

We performed an analysis of variance for the relative grain yield and days to heading of the 122 lines, using winter temperature class and *VrnH1* as sources of variation, to check whether the alleles at *VrnH1* responded similarly or not at winter temperatures. Even though the lines were tested at different years, the use of relative grain yield (as percentage of checks), allowed to combine the results in a single analysis. The fact that there were two different sets of checks reduces the comparability

of the data across the two periods (1 and 2), increasing the error term artificially, but does not invalidate the results. For the two traits, there was significant interaction between winter temperatures and *VrnH1* alleles (Table 4.9).

Table 4.9. Mean squares for relative grain yield and days to heading using as factors the two main alleles of *VrnH1* identified in the advanced lines of the breeding program, across the three classes of winter temperatures 'low', 'intermediate' and 'high'.

	df	Relative yield		df	Days to heading	
VrnH1	1	3250	**	1	132	
Temperature classes	2	2741	**	2	3954	**
***VrnH1*.Temperature classes**	2	1416	**	2	281	*
Residual	1472	194		1312	64	

The lines with *VrnH1-4* yielded less than the ones with the winter allele (*vrnH1*) at the low temperature trials, and reached heading later, but the situation was reversed at higher temperatures, with the lines with Orria allele presenting higher yields and increasingly earlier heading (Fig. 4.3). The relationship between relative grain yield and days to heading for all the lines and their response to temperature confirm these slight, but significant trends (Fig. 4.4). There was a negative relationship between relative grain yield and days to heading for the *VrnH1-4* lines, but it was positive in the lines with the typical winter allele *vrnH1*. Also the lines with *VrnH1-4* presented a negative relationship between temperature and days to heading, and positive relationship between temperature and relative grain yield. But the lines with *vrnH1* presented a negative relationship between temperature and relative grain yield (Fig. 4.4).

For the check cultivars, the interaction of genotype with temperature class was significant for the two periods considered (Table 4.5). Actually, this interaction was more efficient at explaining grain yield GEI per degree of freedom than the AMMI analyses, as indicated by the larger mean squares. The interaction of genotype with temperature class was not stronger than GEI for days to heading (Table 4.5). Looking at the means per genotype and temperature class, winter cultivars Alpha and Barberousse yielded relativey better at the coldest environments, and Graphic relatively worse (Table 4.10). Hispanic, another winter cultivar, had an outstanding performance across temperature classes, but it is endowed with an active *PpdH2* allele, that provides an agronomic advantage even in the absence of full vernalization (Casao et al. 2011).

When looking at individual performances, the winter cultivars produced comparatively better yields under colder than under warmer conditions (Table 4.10). At

the lowest temperatures, it is possible that the strictly winter check cultivars (Barberousse, Alpha, Hispanic) completed their vernalization more timely, and therefore presented not much difference in days to heading and yield compared with the spring cultivars (Graphic, Zaida).

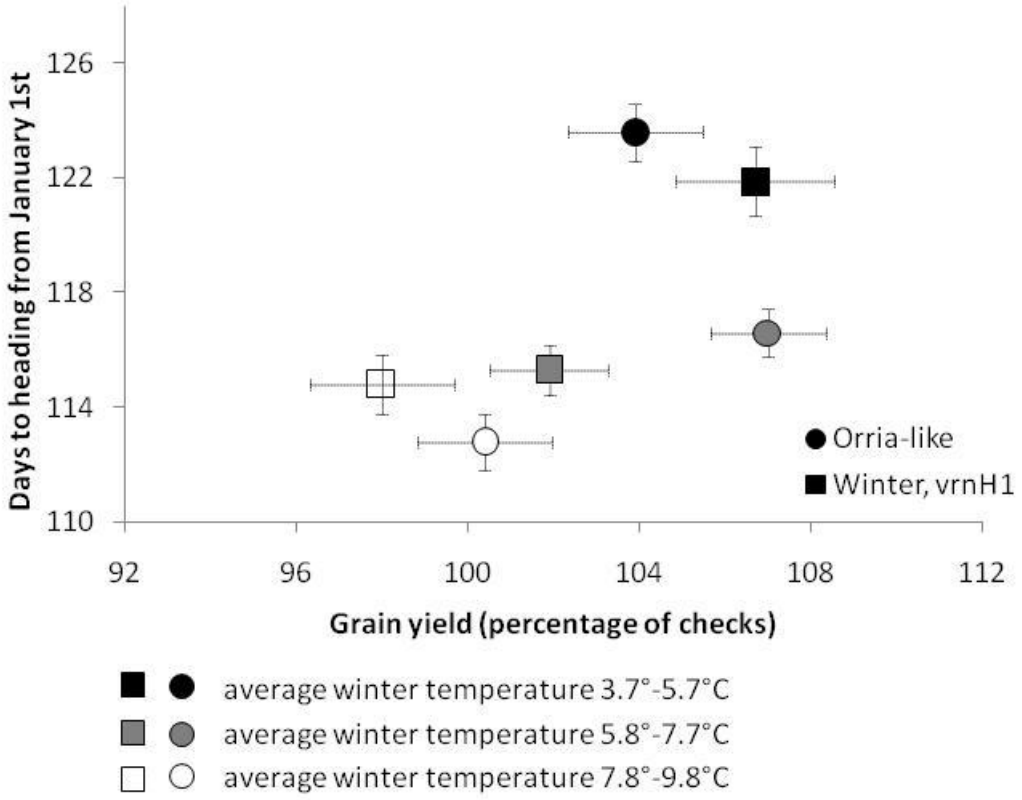

Figure 4.3. The averages of relative grain yield *vs* days to heading of the sets of advanced breeding lines, according to their *VrnH1*alleles (*VrnH1-4* or *vrnH1*), across the field trials divided in classes according to average winter temperatures. Vertical and horizontal segments represent the LSD ($P<0.05$) for days to heading and relative grain yield, respectively.

Table 4.10. Averages of grain yield and days to heading of the check cultivars across the field trials divided in classes according to their average winter temperature.

Period	Genotype	Trials n.	3.7-5.7°C	5.8-7.7°C	7.8-9.8°C
		Grain yield			
2000-04	Alpha	23	5829	4341	5427
	Barberousse	23	6191	4426	5612
	Graphic	23	6027	4362	6328
	Zaida	23	5821	3886	5380
2005-10	Barberousse	40	4069	5894	6534
	Cierzo	40	4393	6265	7556
	Graphic	40	4179	5908	7176
	Hispanic	40	4542	5747	7073
		Days to heading			
2000-04	Alpha	19	119.4	118.7	108.9
	Barberousse	19	120.0	118.6	109.7
	Graphic	19	126.4	120.5	110.3
	Zaida	19	120.2	118.0	108.0
2005-10	Barberousse	38	122.9	115.7	119.8
	Cierzo	38	124.8	117.6	120.2
	Graphic	38	126.5	117.9	119.8
	Hispanic	38	120.3	113.3	115.3

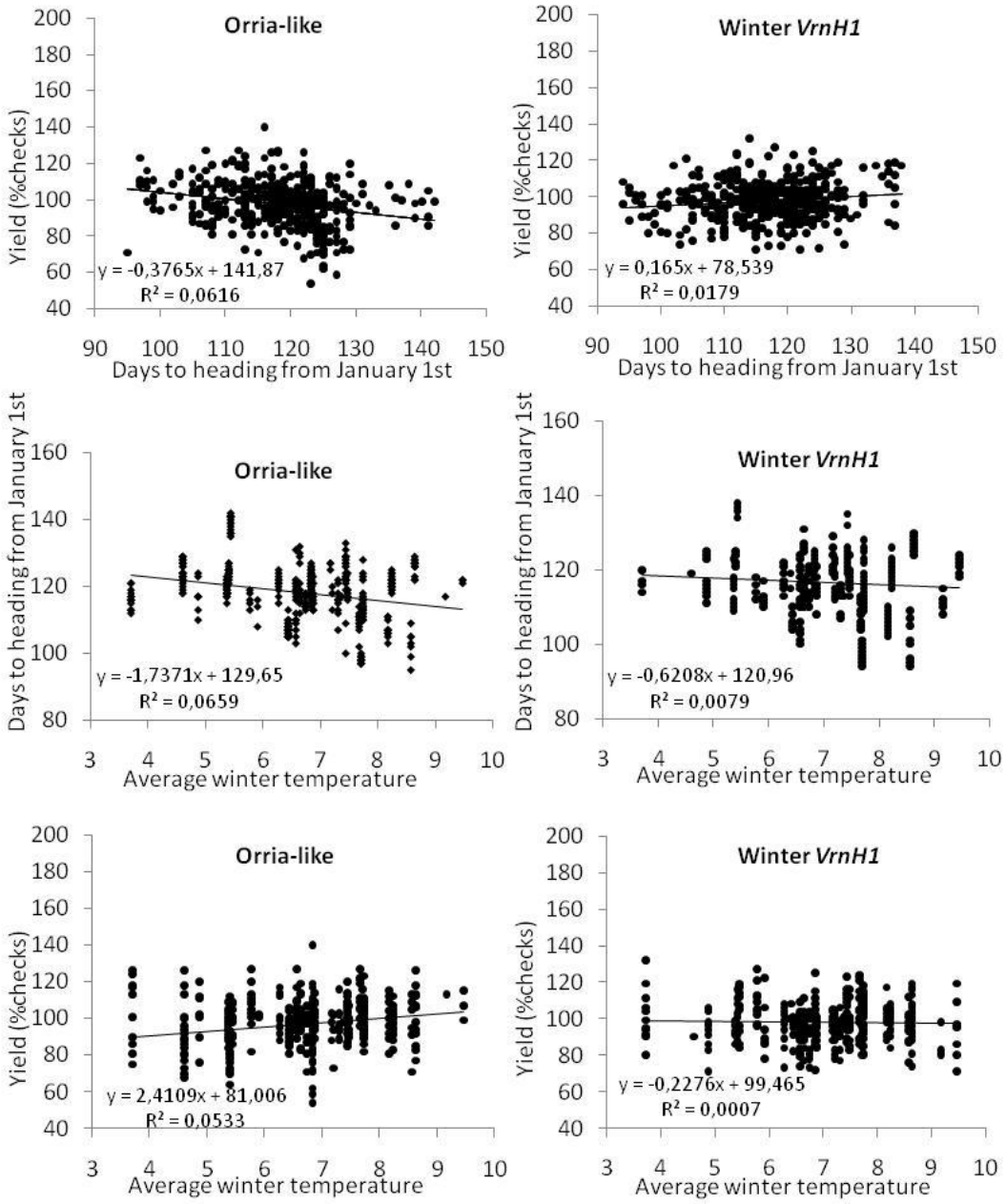

Figure 4.4. Relationship between relative grain yield and days to heading for the lines like Orria in *VrnH1* (*VrnH1-4*) and for the lines that had typical winter *vrnH1*, and their response to temperature during winter.

4.4. Discussion

The large GEI found for grain yield and days to heading, even in analyses with just four genotypes each, is typical of Mediterranean environments (Turner 2004). The patterns of GEI for grain yield were partially influenced by growth habit in the two periods considered. This view was reinforced by the fact that days to heading was similarly affected and, particularly, by the fact that there were significant correlations of the axes apparently related with growth habit, with winter temperatures of the locations, whereas the correlations with other climatic variables were less conspicuous. The placement of Zaida at an intermediate position in the AMMI biplot for the first period, even though it is a spring cultivar, is consistent with its mild vernalization requirement compared to Graphic, which was observed in previous studies (Table 4.11).

Table 4.11. Genetic constitution for vernalization and photoperiod genes in the cultivars under study.

Cultivar	***VrnH1***	***VrnH2***	***PPDH1***	***PPDH2***	**Vern. effect[1]**
Alpha	*vrnH1*	*VrnH2*	*ppdH1*	*ppdH2*	614
Barberousse	*vrnH1*	*VrnH2*	*PPDH1*	*ppdH2*	616
Cierzo	Intermediate	*VrnH2*	*PPDH1*	*ppdH2*	-
Graphic	*VrnH1*	*vrnH2*	*ppdH1*	*PPDH2*	-21
Hispanic	*vrnH1*	*VrnH2*	*PPDH1*	*PPDH2*	755
Zaida	*VrnH1*	*vrnH2*	*ppdH1*	*PPDH2*	235
Orria	Intermediate	*VrnH2*	*ppdH1*	*ppdH2*	111

[1]Vernalization effect is the difference between the thermal time from sowing date to appearance of the last leaf in two treatments: without vernalization and complete vernalization, both under long days (16 h light), taken from Ciudad (2002). The check cultivar Cierzo was not evaluated in the same trial, but we have experimental evidence (not shown) that its vernalization requirement is similar to the one of its parent Orria, whose data are included.

The principal components of the AMMI model of grain yield and days to heading divided the genotypes partially in apparent correspondance with their growth habit, in both periods of study. This fact suggests a relationship between genotype-by-environment and vernalization requirement. Also, the first principal component of the AMMI model for grain yield of period 1, and the second of period 2, were related to the difference in heading dates of the winter and spring cultivars. We hypothesize that these differences are related with the degree of completion of the vernalization needs for the winter cultivars at each particular trial, which was affected by temperature during the winter.

A similar trend was reported by Van Oosterom et al. (1993), who focused on the effect of growth type 'winter' or 'spring' on the GEI. They found that the genotypes were classified into four clusters, related to their growth type and earliness of heading. In our AMMI biplots for grain yield and days to heading, the genotypes were placed according to their growth habit, the winter cultivars being in one section and the spring genotypes in another section, indicating different response of these genotypes to the environmental conditions. Given the limited number of genotypes, we cannot rule out other causes for GEI, but the coincidence is worth noting.

To confirm the results found using only six check cultivars, we used 122 advanced breeding lines, 64 with the *VrnH1-4* allele (as the cultivar Orria) and 58 with the typical winter allele *vrnH1*. The averages of yield and days to heading were calculated for each series of genotypes at each trial using REML. These genotypes were all genetically different, even from different crosses, and the only thing they all had in common was the haplotype at *VrnH1* and *VrnH2*. In consequence, we expect that they do not show a large similarity among them. Our hypothesis is that any similarity in GEI patterns among them should be related to the common haplotype at these genes.

The lines with *vrnH1* and the lines with *VrnH1-4* were different in days to heading and yield between the different trials. The lines with *vrnH1* yielded higher than the *VrnH1-4* lines at low temperature trials and were earlier in heading, but the situation was reversed for both traits in the intermediate and high temperature trials. These observations are consistent with *VrnH1-4* having a small vernalization requirement that, very likely, was fully satisfied even at the trials with intermediate and high winter temperatures. On the contrary, the lines with strict winter *vrnH1* allele need to be exposed to a longer low temperature period to fulfill their vernalization requirement, and may have not been exposed to it but at the lowest temperature trials. All these results suggest that the range of winter temperatures experienced at this sample of Spanish locations and years resulted in a differential response of the genotypes. Also, that these differential responses were related to the allele carried by the genotypes at *VrnH1,* which induced different vernalization requirements, which were not fully met for winter barleys in some Spanish locations.

In a following section of the thesis (chapter 5) we present strong evidence of selection against the strict winter allele at *VrnH1* during the development of a recombinant inbred line (RIL) population from the cross Orria × Plaisant in Lleida (Spain). We detected high distortion of segregation in the region surrounding *VrnH1* on

chromosome 5H, with a much higher frequency of Orria alleles than expected. The explanation was that, although no selection was knowingly applied, there was a strong natural selection against the Plaisant allele at *VrnH1*, which needs a prolonged vernalization period.

It is very likely that other factors besides growth habit and vernalization requirement affected GEI. We already detected a possible relationship of rainfall with GEI in period 2, possibly as the result of differential reaction of other genes, of unknown nature. Even the type of spike may have an effect according to the AMMI biplots for grain yield and days to heading of period 2 (Fig. 4.1), because the first component divided the genotypes into two sections according to the row type: the two-row cultivars Graphic and Hispanic to the right, and the six-row cultivars Cierzo and Barberousse to the left. A similar trend was reported by Bensemane et al. (2011), who focused on the phenotypic variation within two- and six-rowed barley breeding lines grown under semi-arid conditions. They found that the first two principal components separated the two groups of lines into groups according to row-type, explaining jointly 70.31% of total variation. In another study, Garcia del Moral et al. (2003) studied yield stability in two and six-rowed barleys under Mediterranean conditions. They found that there was no GEI within each row type, while differences between two and six rowed barley for grain yield and its components changed from one environment to another. There was a noticeable difference between two and six rowed barley cultivars in their response to environmental conditions, where the two-rowed barleys were more responsive to environmental changes than their six-rowed counterparts, which consistently showed more stable behavior.

From these results we can conclude that the genotype-by-environment interaction for grain yield and days to heading of barley are influenced by growth habit under the Spanish conditions. And that the variable winter temperatures occurring across the Spanish barley growing areas lead to differential responses of the genotypes according to their growth habit. This finding indicates that the alleles of *VrnH1* can be managed in barley breeding to fine tune the cultivars to prevailing winter temperatures.

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Chapter 5

QTL for agronomic traits in an elite barley population for Mediterranean conditions

5.1 Introduction

Breeding for yield stability in the Mediterranean environments has been slow due to the high variability in timing, duration and the severity of a number of climatic stresses (Baum et al. 2003). Consequently, the most difficult task for cereal breeders in Mediterranean countries is to develop varieties able to tolerate drought stress fluctuating across years and environments, by improving yield-stability. In the Mediterranean area, crop performance is usually related with the response to abiotic stresses (Teulat et al. 2001). Although there have been a number of studies dealing with barley breeding issues for such environments (Ceccarelli et al. 2007, and references therein), barley breeding has made little progress in stress-prone areas (Pswarayi et al. 2008). Therefore, there is still a need for studies addressing barley productivity in Mediterranean conditions. The intrinsic interest of this area of research is enhanced by the current and future effects of climate change on agricultural production which, in a number of Mediterranean countries, are already causing farmers to change cropping from wheat to barley due to the latter's greater abiotic stress tolerance (Comadran et al. 2008).

New approaches have to be taken into account for the dissection of the genetic mechanisms underlying the tolerance to abiotic stresses. The Mediterranean basin conditions present particular conditions regarding sowing dates, and environment variables such as high light intensity, high temperatures and evaporative demand, and lower rainfalls, all of which are erratically distributed (Loss and Siddique 1994).

Studies aiming at the identification of QTLs for yield and its components in barley are quite abundant in the literature. But QTL for grain yield in barley are an elusive target, as many are affected by large QTL×Environment interaction (Romagosa et al. 1999), and thus are not suitable target for marker assisted selection (MAS). Given the difficulty to find stable QTLs for yield, some authors claim that the improvement of yield in Mediterranean conditions will probably come through a combination of stable QTLs involved in the expression of traits significantly correlated with yield (Teulat et al. 2001). It has also been suggested that yield QTLs in cereals are not easily transferable between regions and also between plant materials. For this reason, the search for QTL with immediate potential for application should be carried out as close as possible to the target environments, and with plant materials closely related to the germplasm used in the breeding programs.

We developed a population of recombinant inbred lines (RILs) from a cross between two elite barley cultivars, Orria and Plaisant. This cross has resulted in a large number of lines reaching the final stages of the Spanish National Barley Breeding Program, and has been a source of successful new cultivars in recent years characterized by a wide range of adaptation across the Spanish environments. The objective of this study is to investigate the genetic factors that underlie the advantageous traits found in this cross, to facilitate the design of new breeding strategies, and the implementation of marker assisted selection for Mediterranean conditions.

5.2 Materials and methods

Plant materials

The cross between two six-row parents, Orria and Plaisant, has proved to be one of the best crosses of the Spanish National Barley Breeding Program. Orria ((((Api × Kristina) × M66.85) × Sigfrido's) × 79W40762), a semi-dwarf cultivar selected in Spain from a CIMMYT nursery, is a facultative cultivar, that is highly productive across most regions in Spain and has a very mild vernalization requirement. Plaisant (Ager × Nymphe) is a French cultivar with strict winter growth habit; whilst it is less productive in Spain, it is one of the few European six-row winter cultivars with acceptable malting quality and consequently was a popular cultivar in Spain. We derived a total of 330 RILs from the Orria × Plaisant cross by selfing a single plant for each segregating generation up to and including F7. The product of each F7 plant was then multiplied and a subset of 217 RILs was used for genotyping, from which a further subset of 120 RILs was randomly chosen for phenotyping.

Field trials

Five trials were carried out at four locations: Sádaba, (Zaragoza, Spain) during the 2008-2009 and 2009-2010 seasons, Gimenells, (Lleida, Spain) in 2008-2009, Bell-lloc, (Lleida, Spain) in 2009-2010, and Fiorenzuola d'Arda (Piacenza, Italy), in 2010 (Table 5.1).

Table 5.1. Description of the field trials.

Location	Province-Country	Code	Latitude	Longitude	Season	Sowing date
Gimenells	Lleida-Spain	L09	41°39′N	0°23′E	2008/2009	01/12/2008
Bell-lloc	Lleida-Spain	L10	41°37′N	0°46′E	2009/2010	02/11/2009
Sádaba	Zaragoza-Spain	Z09	42°17′N	1°16′W	2008/2009	22/11/2008
Sádaba	Zaragoza-Spain	Z10	42°17′N	1°16′W	2009/2010	26/11/2009
Fiorenzuola d'Arda	Piacenza-Italy	F10	44°56′N	9°54′E	2009/2010	01/03/2010

Due to unfavourable weather conditions during the 2009 fall at Fiorenzuola d'Arda, this trial was sown very late, on March 1st. The two Lleida locations are less than 50km apart and climatically very similar and can therefore be considered as the same location. The experimental design at each trial was an alpha lattice with three replicates, each arranged in 8 incomplete blocks of 15 entries per incomplete block. Plots at Sádaba consisted of 4 rows, 2.7 m long, and 20 cm between rows. At Gimenells and Bell-lloc, each plot consisted of 8 rows 2.5 m long and a spacing of 15 cm between rows. In Fiorenzuola, the individual plot consisted of 8 rows, 15 cm apart and 3 m long. In all trials, sowing density was set to 1050 seeds per plot. Crop management followed local practices at each location. Climatic conditions, monthly average minimum and maximum temperatures for the testing locations are shown in Figure 5.1.

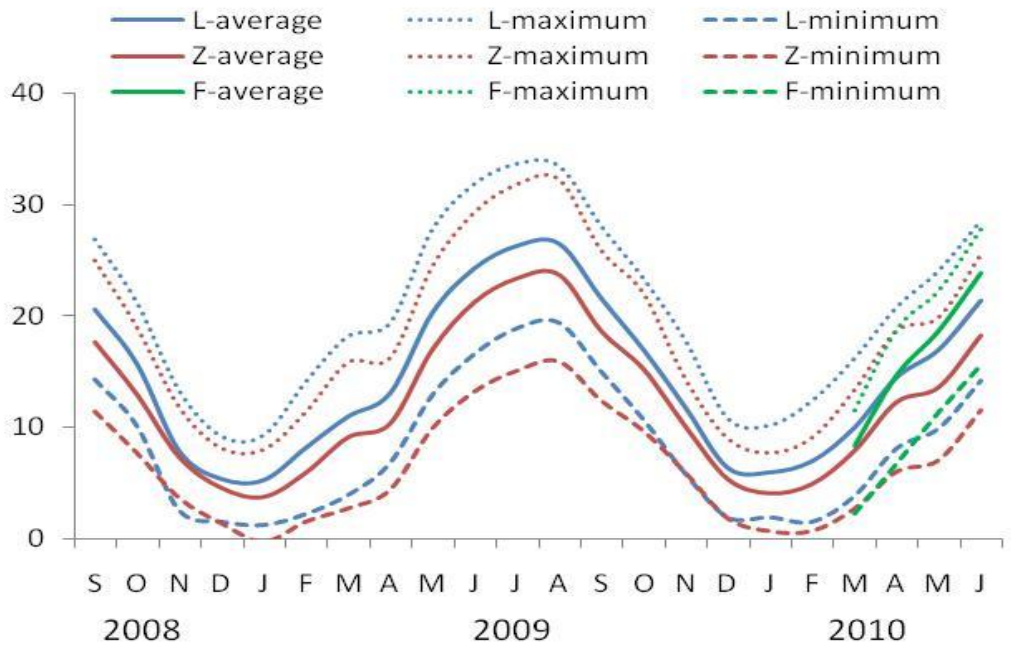

Figure 5.1. Monthly average, minimum and maximum temperatures for the testing locations in Lleida (L), Zaragoza (Z), and Fiorenzuola d'Arda (F) during the field seasons 2008-09 and 2009-10.

Plots were scored for: grain yield, days to heading, plant height, maturity time, thousand grain weight, hectolitre weight, grain length, grain width, grain area, early vigour, growth habit, susceptibility to powdery mildew and spot blotch but not all traits were recorded in all five trials (Table 5.2).

Table 5.2. Traits measured or recorded at each of the five field trials.

Trait	Code	Units	F10	L09	L10	Z09	Z10
Grain yield	YLD	kg ha^{-1}	x	x	x	x	x
Days to heading	DHE	Days	x	x	x	x	x
Plant height	PHE	cm	x	x	x	x	x
Maturity time	MAT	Days	x	x	x		
Thousand grain weight	TGW	G		x	x	x[a]	x[a]
Hectolitre weight	HEC	kg hl^{-1}		x	x	x[a]	x[a]
Grain length	LEN	Mm				x[a]	x[a]
Grain width	WID	Mm				x[a]	x[a]
Grain area	ARE	mm^2				x[a]	x[a]
Early vigor	VIG	scale 1 to 3[b]			x		x
Growth habit	GRW	scale 1 to 3[c]			x		
Powdery mildew	POW	scale 0 to 9[d]	x				
Spot blotch	SPO	scale 0 to 9[d]	x				

[a] one replication
[b] 1, less vigor; 3 maximum vigor
[c] 1, prostrate; 2, intermediate; 3, erect
[d] 0, no disease symptoms; 9, maximum expression of disease symptoms

Grain yield was measured as the weight of grain combine harvested per plot and converted to kilograms per hectare by taking the harvested plot area into account. Days to heading were recorded as the number of days between January 1st and the date when approximately 2 cm of awns were visible on 50% of the stems in each plot (Decimal Growth Stage 49). Plant height was measured in centimetres from the ground to the top of the stalk (excluding the spike). Maturity time was defined as the number of days between January 1st and the day when approximately 50% of spikes had ripened (turned to yellow, Decimal Growth Stage 91). Thousand grain weight was estimated from the weight of a sample of 1000 grains. Hectolitre weight was calculated with a Dickey-John analyser model GAC-II. A Marvin Digital Seed Analyzer (GTA Sensorik GmbH) was used to estimate the average grain length, width and area from a 22 cm3 sample of seed. Growth habit and early vigour were visually scored, using a scale from 1 (prostrate or

poor vigour, respectively) to 3 (erect growth or excellent vigour, respectively). Powdery mildew (*Blumeria graminis f.sp. hordei*) and spot blotch (*Cochliobolus sativus*) were rated using a 0 to 9 scale in which 0 represented no disease symptoms, and 9 was more than 90 percent of leaf tissue diseased.

Statistical analysis of field trials

The alpha-lattice design was used to produce adjusted means for all traits scored on each individual trial by using the linear mixed model analysis implemented in the REML directive in Genstat 14 (VSN International 2011) to account for spatial differences detected by the incomplete blocks. Genotypes were fitted as a fixed factor and all other effects were considered random. The joint analysis across environments was done on these REML averages. The overall error mean square was calculated as the average of the error mean squares at each individual trial, and added as the residual term to the joint analysis. To account for the loss of the replicates in this analysis, the sums of squares for genotypes, environments and genotype-by-environment were multiplied by 3. This analysis was done for grain yield, days to heading and plant height for the five trials; for maturity time for trials L09, L10 and F10; and for thousand grain weight and hectolitre weight just at the two Lleida trials L09 and L10.

Genotyping

Genomic DNA was extracted from leaf samples obtained from 14-days old individual seedlings of the 217 RILs and the two parents. Genotyping was carried out at the Southern California Genotyping Consortium, using the Illumina GoldenGate Bead array platform Barley Oligo Pooled Array 1, which analyses 1536 genome wide SNPs (Close et al. 2009). PCR specific markers for genes *VrnH1* (von Zitzewitz et al. 2005) and *PpdH1* (Turner et al. 2005) were also assayed in the 217 RILs using the primers and protocols described by the authors.

Map construction and QTL mapping

JoinMap 4 (van Ooijen 2006) was used for map construction. As the map locations of most of the 1536 SNPs was known, we chose a LOD grouping threshold

that divided the markers into the appropriate chromosomal groups, although this meant that some chromosomes were fragmented into two or more groups. For each linkage group so formed, the maximum likelihood mapping algorithm was used, in a first step, to estimate the best marker order within it. The distances between markers, using Kosambi's mapping function, were then recalculated using the regression mapping algorithm in a second step but markers that were discarded after the second round of Joinmap 4 were excluded from the final map.

QTL × Environment analysis was performed with the multi-environment routine for linkage mapping implemented in Genstat 14. The genotypic data and maps produced by Joinmap 4 were used to estimate genetic predictors for each marker locus and at 2 cM intervals where gaps between adjacent markers were greater than 2cM. After choosing the best variance–covariance model for each trait, we used simple interval mapping scan to identify an initial set of cofactors for use in iterative rounds of composite interval mapping until there was no change in the cofactors. The final set of cofactors was used in a multi-environment mixed model to test whether each represented a QTL main effect or a QTL × Environment and estimate allelic effects at each environment. In all QTL analyses, we used the Li and Ji method to estimate a 5% genome-wide significance threshold for the -log10 (P) values. The minimum cofactor distance was set to 30 cM, and the minimum distance to declare independent QTLs was set to 20 cM.

5.3 Results

Field experiments

Despite the phenotypic similarity of the parents, considerable transgressive segregation was observed at all sites at which variates were scored and the population extremes were generally significantly better or worse than the higher or lower scoring parent, respectively (Table 5.3). Orria generally had a higher yield and a lower plant height and hectolitre weight than Plaisant. The differences between the parents for heading date were significant only at the Lleida and Fiorenzuola locations, with Plaisant later than Orria. L09 and L10 had greater overall growth (as suggested by larger plant height) and yield potential than the other three trials, together with earlier heading but lower thousand grain weight.

Table 5.3. Descriptive statistics (mean, minimum, maximum, standard deviation and coefficient of variation) for the agronomic traits observed in the parents (Orria, Plaisant) and in the population of 112 RILs.

	Parents		**Recombinant inbred lines**				
	Orria*	Plaisant	Mean	Min	Max	SD	CV
L09							
YLD (kg ha^{-1})	5848 [a]	5879 [a]	5543	3390	7619	620	11.2
DHE (days)	105.7 [a]	107.7 [b]	107.7	99.0	113.0	2.3	2.1
PHE (cm)	108.3 [a]	118.3 [b]	113.4	95.0	135.0	7.5	6.6
MAT (days)	143.0 [a]	145.0 [b]	144.9	140.0	149.0	1.6	1.1
TGW (g)	30.4 [a]	34.4 [b]	33.3	23.3	45.2	4.2	12.6
HEC (kg hl^{-1})	67.7 [a]	72.4 [b]	70.5	59.9	78.1	3.0	4.3
L10							
YLD (kg ha^{-1})	7143 [a]	6095 [b]	6329	4343	7867	607	9.6
DHE (days)	111.3 [a]	114.0 [b]	112.9	106.0	119.0	2.2	2.0
PHE (cm)	97.0 [a]	101.3 [a]	99.2	64.0	118.0	8.5	8.5
MAT (days)	148.0 [a]	149.7 [a]	149.8	147.0	156.0	2.4	1.6
TGW (g)	36.4 [a]	34.6 [a]	38.4	23.2	48.5	4.7	12.3
HEC (kg hl^{-1})	69.4 [a]	72.2 [b]	70.5	59.7	75.6	2.8	3.9
VIG (scale 1 to 3)	3.0 [a]	2.0 [b]	2.4	1.0	3.0	0.6	24.1
GRW (scale 1 to 3)	1.7 [a]	3.0 [b]	2.3	1.0	3.0	0.8	33.8

*Values followed by the same letter are not significantly different from 0 According to an LSD ($P<0.05$).

Table 5.3. (Continued)

	Parents		Recombinant inbred lines				
	Orria	Plaisant	Mean	Min	Max	SD	CV
Z09							
YLD (kg ha^{-1})	3964[a]	2631[b]	3302	1982	4360	371	11.3
DHE (days)	122.3[a]	121.7[a]	122.3	116.0	129.0	2.6	2.1
PHE (cm)	70.7[a]	85.7[b]	73.6	61.0	94.0	5.7	7.8
TGW (g)	38.5	42.4	41.2	33.1	47.9	3.1	7.6
HEC (kg hl^{-1})	69.4	73.6	71.6	67.2	75.8	1.8	2.5
LEN (mm)	8.6	8.1	8.3	7.3	9.2	0.4	4.7
WID (mm)	3.1	3.2	3.1	2.9	3.5	0.1	3.2
ARE (mm^2)	20.8	20.1	20.4	17.3	23.0	1.1	5.5
Z10							
YLD (kg ha^{-1})	4174[a]	3015[b]	3641	2306	4613	375	10.3
DHE (days)	116.7[a]	116.0[a]	116.5	112.0	123.0	1.8	1.6
PHE (cm)	71.7[a]	87.7[b]	78.5	61.0	95.0	6.0	7.7
TGW (g)	39.9	37.1	39.7	30.0	52.0	3.9	9.8
HEC (kg hl^{-1})	65.2	69.7	66.8	59.2	71.9	2.9	4.4
LEN (mm)	8.5	7.9	8.6	7.4	10.1	0.7	7.8
WID (mm)	3.0	3.1	3.1	2.8	3.4	0.1	3.5
ARE (mm^2)	19.6	18.9	20.2	16.9	23.5	1.4	6.9
VIG (Scale 1 to 3)	2.3[a]	2.7[a]	2.3	1.0	3.0	0.5	23.4
F10							
YLD (kg ha^{-1})	5517[a]	3433[b]	3775	360	5540	885	23.4
DHE (days)	144.3[a]	147.3[b]	144.9	135.0	165.0	5.3	3.6
PHE (cm)	70.0[a]	66.7[a]	65.9	50.0	80.0	5.6	8.4
MAT (days)	169.3[a]	170.3[a]	169.7	163.0	185.0	4.7	2.8
POW (scale 0 to 9)	3.7[a]	6.3[b]	5.7	1.0	8.0	1.4	23.7
SPO (scale 0 to 9)	0.7[a]	6.0[b]	2.0	0.0	8.0	2.1	104.1

*Values followed by the same letter are not significantly different from 0 According to an LSD ($P<0.05$).

The over-sites analysis revealed not only that there were significant main effects of genotype and site for the six traits measured at all five trials but also that there were significant genotype × site interactions for all, although the mean square for the latter was much less than that for genotype (Table 5.4).

Table 5.4. Analysis of variance for the agronomic traits measured in five trials, for a population of 112 RILs from the cross Orria×Plaisant (degrees of freedom, df and mean squares, ms are presented).

	YLD			DHE			PHE			TGW			HEC			MAT		
Source of var.	df	ms		Df	ms		df	ms		df	ms		df	ms		df	ms	
Environment (E)	4	600325650	**	4	70293.9	**	4	129160.3	**	3	3977.5	**	3	1436.6	**	2	58032	**
Genotype (G)	111	1251084	**	111	80.4	**	111	319.3	**	111	141.4	**	111	55.6	**	111	45	**
G×E	444	638082	**	444	14.2	**	444	35.0	**	333	12.7	**	333	7.1	**	222	17.7	**
VrnH1×E	5	6604161	**	5	185.3	**	5	107.8	**	4	2.9	n.s	4	12.3	n.s	3	221.9	**
Residual	439	570132		439	12.3		439	34.2		329	12.8		329	7.1		219	14.9	
Error	1258	116733		1258	0.6		1258	15.5		1008	3.9		1008	1.9		756	1.6	

** significant for p <0.01
n.s. non significant

We utilised the linear correlation coefficients between grain yield, days to heading, plant height and thousand grain weight of the RILs within each trial to interpret the dynamics of grain yield variation across environments (Table 5.5).

Table 5.5. Correlation coefficients between some agronomic traits in 112 RILs of the Orria × Plaisant cross, in five field trials.

	DHE YLD	**PHE YLD**	**TGW YLD**	**DHE TGW**	**PHE TGW**
L09	-0.47 **	-0.18	0.45 **	-0.22 *	0.31 **
L10	-0.46 **	0.19 *	0.41 **	-0.24 *	0.60 **
Z09	0.01	-0.26 **	-0.09	-0.16	0.43 **
Z10	0.03	-0.35 **	-0.08	-0.27 **	0.33 **
F10	-0.77 **	0.37 **			

* and ** indicate significant (p <0.05) and highly significant (p <0.01) respectively

The correlation between days to heading and yield was not significant in Z09 and Z10 (i.e., production was independent of cycle length) but was significant and negative in L09, L10 and F10, meaning that later lines produced lower yields. The correlation between thousand grain weight and grain yield was not significant in Z09 and Z10 but was significant and positive in L09 and L10. The correlation between plant height and yield was significant in four of the five trials, but with opposite signs, negative in Z09 and Z10, and positive in L10 and F10. Other coefficients were more conserved across trials, like the correlation between thousand grain weight and both days to heading (negative) and plant height (positive).

A principal component analysis of these variables, based on the correlations among them, offers a better insight on the relationships within and between traits. Days to heading, plant height and thousand grain weight were rather closely correlated across the trials (Fig. 5.2). All the points corresponding to each trait were placed in the same quadrant of the graph of the loadings on the first two principal components. These two components together explained 54% of the total variance. Grain yield data points, however, were distributed over two quadrants, indicating changes in the direction of correlations within this trait and among traits.

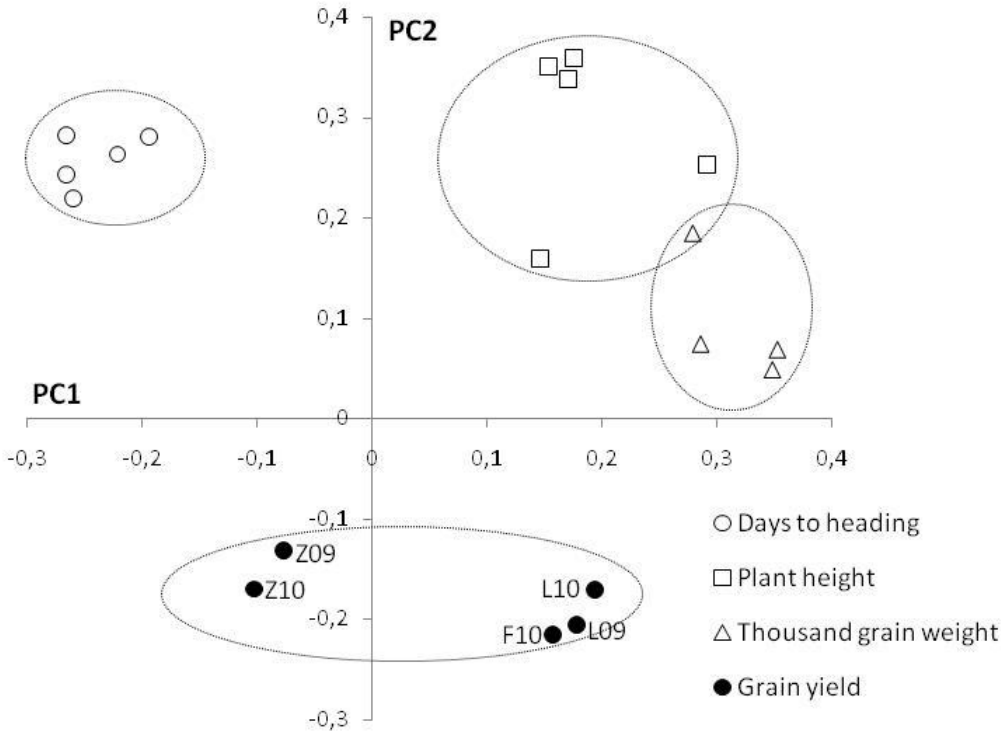

Figure 5.2. Plot of the first two axis of a principal component analysis carried out with the variables days to heading, plant height, thousand grain weight and grain yield, measured at five field trials.

Genetic map

Out of the 1536 SNPs assayed, monomorphic markers, markers with more than 10% missing data and those with low quality scores (GenTrain score below 0.45) were removed from the data set. Excessive marker redundancy was reduced in a second round, resulting in a total of 384 high-quality markers being used for map construction. These markers formed 13 linkage groups at a LOD score of 7 with chromosomes 1H, 3H, 6H and 7H represented by one group and chromosomes 2H, 4H and 5H fragmented into 3, 2 and 4 groups respectively (Fig. 5.3). After ordering the markers, comparison of our map with other consensus maps (Close et al 2009; Muñoz-Amatriain et al. 2011) showed good correspondence of marker order in all linkage groups.

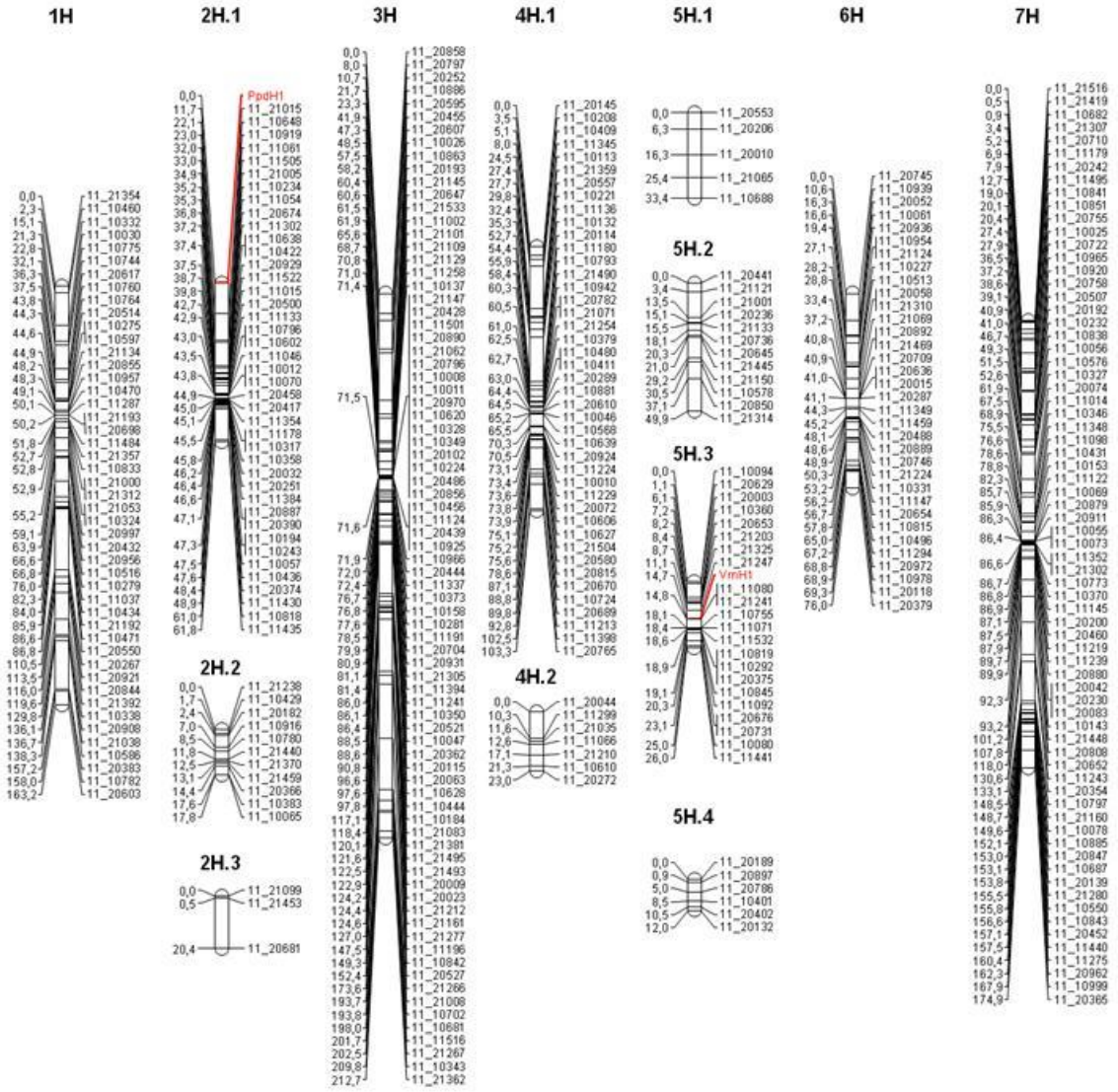

Figure 5.3. Linkage map of the RIL population from the cross between barley cultivars Orria and Plaisant. Map positions are given in centimorgans (cM), using the Kosambi function. The two major flowering time genes segregating in this population are highlighted in red font.

PpdH1 was the most distal marker on the short arm of 2H with 11_21015 being the closest SNP to it. 11_21015 maps close but proximal to the BOPA2 markers 12_30871 and 12_30872 (Muñoz-Amatriain et al. 2011), which are SNPs in *PpdH1* so the position of *PpdH1* is consistent with previous reports. The *PpdH1* SNPs are located at 25.3 cM on the consensus map of Muñoz-Amatriain et al. (2011) but the distal region of 2HS is not polymorphic in Orria × Plaisant. *VrnH1* was mapped on the long arm of 5H between SNPs 11_21247 and 11_11080, which is precisely where SNP 12_30883, a SNP in *VrnH1*, maps on the consensus map of Muñoz-Amatriain et al. (2011), indicating that this developmental gene is also correctly located.

Among the 384 mapped markers, 288 segregated close to the expected 1:1 ratio. But 55 markers in 1H, 2H.1, 3H, 4H.1, and 6H presented distorted segregation towards Plaisant (based on a chi-squared test for $P<0.01$). On the other hand, 19 markers scattered over 2H.3, 3H, 4H.1 and 7H, and all 23 markers on 5H.3 showed distorted segregation towards Orria alleles (Fig. 5.4).

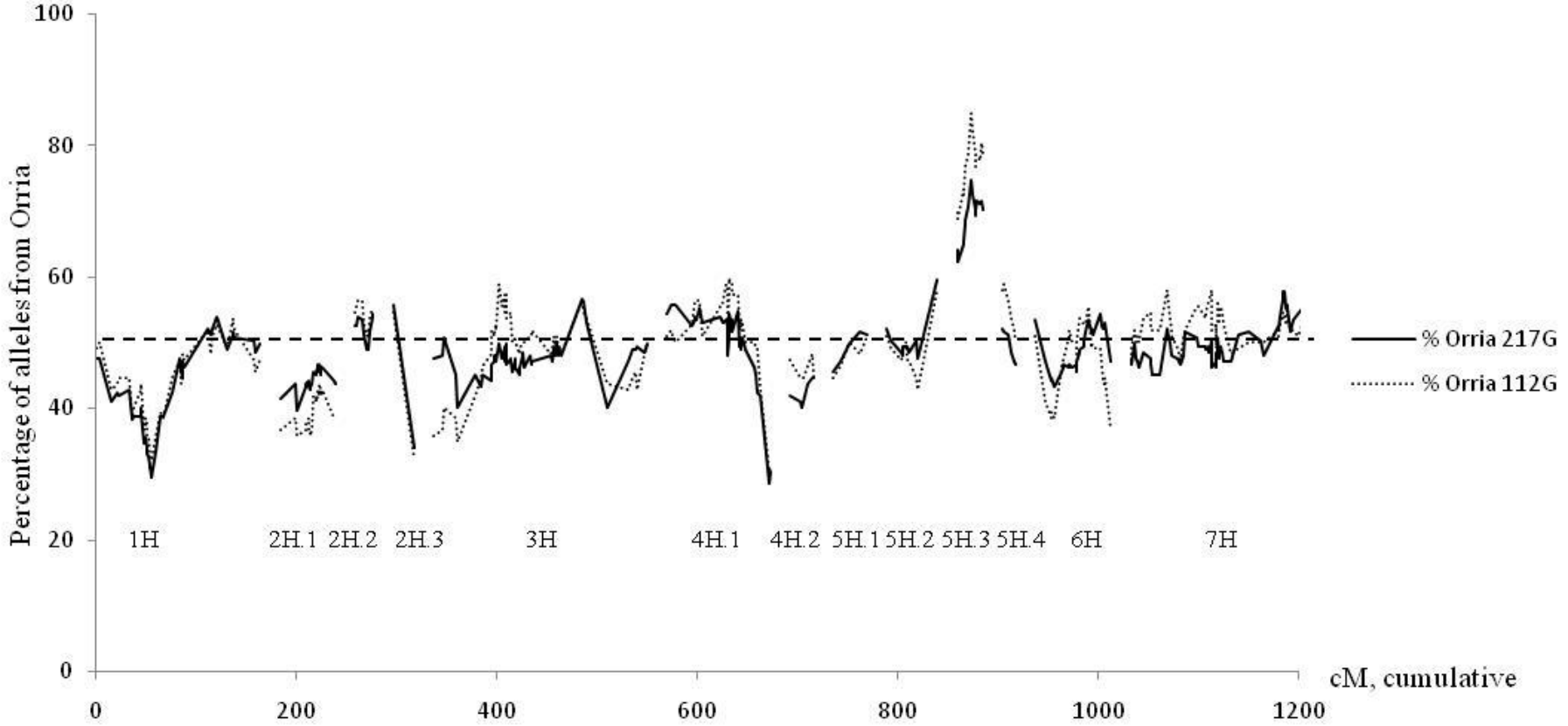

Figure 5.4. Segregation distortion in the Orria × Plaisant RIL population. Percentage of alleles from Orria in 112 field tested genotypes or 217 genotyped RILs.

QTL analyses

QTLs were found for all traits, except for grain length, early vigour, growth habit and spot blotch tolerance. A total of thirty-three QTLs were detected for the traits under study but 23 were not consistent across locations as they were detected as interactions with the environment, although significant cross-over interactions were only detected for three of them.

Four QTLs for grain yield were identified on 1H, 2H.1, 5H.3, and 7H (Table 5.6). Whilst all considerably exceeded the significance threshold, all showed significant interactions with the environment.

The most significant was the QTL located on chromosome 5H.3, at the *VrnH1* locus, in which Plaisant alleles reduced grain yield significantly at three trials, but were not significant at Z09 or Z10. This cross-over interaction QTL had a strong additive effect of -591.8 kg ha-1 at F10 and explained 49.8% of the phenotypic grain yield variation at this trial. Similarly, the Plaisant allele at the QTL located between SNPs 11_10327 and 11_20074 on chromosome 7H, significantly reduced grain yield at the same three trials with the greatest effect again at F10, but was also not significant at the two Zaragoza trials. The second most significant QTL was detected on chromosome 2H.1 between SNPs 11_11430 and 11_10818 and was significant at all four Spanish sites but exhibited a strong cross-over interaction between Zaragoza, where the Plaisant allele reduced yield, and Lleida, where the same allele increased yield. The fourth QTL was located at SNPs 11_10275 and 11_10597, which are co-located on chromosome 1H. Whilst it was only significant at two sites, it was again a cross-over interaction with the Plaisant allele decreasing yield at one Zaragoza site but increasing yield at one Lleida site.

Table 5.6. QTLs for agronomic traits detected by composite interval mapping in the RILs of 'Orria' × 'Plaisant' cross in the five trials.

						Additive effect					% Explained variance					
Trait	**SNP**	**Chr.**	**Pos.**	**Conf. Int.**	**-log10 (P)**	**L09**	**L10**	**Z09**	**Z10**	**F10**	**L09**	**L10**	**Z09**	**Z10**	**F10**	**QTL×E**
YLD	11_10275	1H	44.6	37.5 - 46.6	4.7	121.9 *	-40.0	26.7	-79.6 *	-3.0	9.1	0.7	1.0	7.4	0.0	<0.001
	11_11430	2H.1	54.1	48.4 - 61.8	5.7	80.8 *	126.9 *	-90.0 *	-71.8 *	-148.3	4.0	6.6	10.9	6.0	3.1	<0.001
	VrnH1	5H.3	14.8	11.1 - 18.1	6.9	-100.9 *	-138.2 *	-68.4	30.4	-591.8 *	6.2	7.9	6.3	1.1	49.8	<0.001
	11_10327	7H	58.2	51.5 - 67.5	5.4	-173.3 *	-99.9 *	34.3	-1.1	-205.7 *	18.4	4.1	1.6	0.0	6.0	<0.001
DHE	*PpdH1*	2H.1	5.9	0.0 - 9.8	24.7	0.3	0.4	-1.3 *	-0.5 *	-2.7 *	2.1	3.2	26.4	8.6	26.4	<0.001
	VrnH1	5H.3	14.8	11.1 - 18.1	12.0	1.0 *	1.2 *	1.1 *	0.8 *	3.7 *	20.9	28.2	18.7	17.8	49.8	<0.001
	11_10327	7H	58.2	52.6 - 67.5	5.4	0.7 *	0.7 *	0.7 *	0.7 *	0.7 *	11.7	11.4	9.3	17.2	2.0	n.s
PHE	*PpdH1*	2H.1	3.9	0 - 13.5	4.2	0.6	0.2	-0.4	-0.3	-1.7 *	1.0	0.1	0.7	0.4	13.3	<0.001
	11_11505	2H.1	33.0	24.8 - 35.2	3.8	-0.1	0.7	1.3 *	0.5	-0.4	0.0	1.1	7.8	0.8	0.8	<0.001
	11_10379	4H.1	62.5	61.0 - 62.7	5.6	-0.4	-1.6 *	-0.2	-0.7	-1.7 *	0.4	5.4	0.1	1.7	13.6	<0.001
	11_10954	6H	25.2	19.4 - 28.2	6.8	2.9 *	2.5 *	1.8 *	2.5 *	0.8	23.2	13.6	13.3	22.5	2.8	<0.001
	11_20200	7H	87.1	82.3 - 93.2	4.1	1.1 *	1.1 *	1.1 *	1.1 *	1.1 *	3.6	3.0	5.7	4.7	6.4	n.s
MAT	11_21015	2H.1	13.5	5.9 - 18.6	10.6	-0.1	-0.1	-	-	-2.3 *	0.2	0.1	-	-	24.9	<0.001
	11_20850	5H.2	35.4	30.5 - 42.6	3.8	0.5 *	0.5 *	-	-	0.5 *	12.9	5.5	-	-	1.2	n.s
	VrnH1	5H.3	14.7	12.9 - 16.5	16.9	0.7 *	1.1 *	-	-	3.4 *	27.9	25.9	-	-	57.1	<0.001
	11_10327	7H	58.2	46.7 - 67.5	4.7	0.5 *	0.5 *	-	-	0.5 *	13.2	5.6	-	-	1.2	n.s

¥ % Phenotypic variance explained by detected QTLs
* indicate significant (p <0.05)
n.s. non significant

Table 5.6. (continued)

						Additive effect					%Expl. var.¥					
Trait	**SNP**	**Chr.**	**Pos.**	**Conf. Int.**	**-log10 (P)**	**L09**	**L10**	**Z09**	**Z10**	**F10**	**L09**	**L10**	**Z09**	**Z10**	**F10**	**QTL×E**
TGW	11_10379	4H.1	62.5	61.0 - 62.7	6.0	-1.3 *	-1.3 *	-1.3 *	-1.3 *	-	11.4	8.3	16.6	10.8	-	n.s
	11_10610	4H.2	21.3	14.1 - 23.0	5.2	1.0 *	0.5	0.9 *	-0.1	-	7.4	1.4	7.3	0.1	-	<0.001
	11_20892	6H	40.8	37.2 - 41.1	3.9	0.9 *	1.3 *	0.1	0.8 *	-	5.7	9.2	0.2	4.7	-	<0.001
HEC	11_20267	1H	112.0	104.6 - 113.5	14.5	0.4	1.0 *	0.8 *	1.5 *	-	2.3	15.7	21.4	26.2	-	<0.001
	11_10818	2H.1	57.6	50.6 - 61.8	7.7	0.9 *	0.9 *	0.9 *	0.9 *	-	11.1	11.8	23.9	9.3	-	n.s
	11_21440	2H.2	11.8	3.9 - 14.4	4.0	0.4	-0.4	-0.1	-0.1	-	2.1	1.8	0.4	0.2	-	<0.001
	11_21362	3H	212.7	202.5 - 212.7	3.8	0.7 *	0.1	0.4 *	0.6 *	-	6.9	0.1	5.8	3.6	-	<0.001
	11_20010	5H.1	18.1	8.3 - 25.4	5.6	0.7 *	0.6 *	0.1	0.8 *	-	6.9	5.5	0.1	7.4	-	<0.001
	11_20074	7H	63.8	58.2 - 67.5	11.1	-0.8 *	-0.8 *	-0.2	-1.2 *	-	9.9	10.3	0.8	17.5	-	<0.001
WID	*PpdH1*	2H.1	3.9	0 - 11.7	5.3	-	-	0.04 *	0.04 *	-	-	-	13.3	11.8	-	n.s
	11_10379	4H.1	62.5	60.5 - 62.7	5.5	-	-	-0.04 *	-0.04 *	-	-	-	12.6	11.2	-	n.s
	11_20441	5H.2	0	0 - 5.1	5.4	-	-	0.04 *	0.002	-	-	-	11.9	0	-	<0.001
ARE	11_20267	1H	104.6	94.7 - 124.7	5.2	-	-	-0.5 *	-0.5 *	-	-	-	20.1	13.2	-	n.s
	11_10379	4H.1	62.5	60.5 - 62.7	3.9	-	-	-0.4 *	-0.4 *	-	-	-	11.7	7.6	-	n.s
POW	11_10383	2H.2	17.6	7.0 - 17.8	4.0	-	-	-	-	0.3 *	-	-	-	-	9.5	-
	11_20924	4H.1	70.5	67.1 - 77.1	6.3	-	-	-	-	-0.4 *	-	-	-	-	16.1	-
	11_10576	7H	50.4	44.8 - 58.2	4.4	-	-	-	-	0.3 *	-	-	-	-	11.0	-

¥ % Phenotypic variance explained by detected QTLs
* indicate significant (p <0.05)
n.s. non significant

Three QTLs for days to heading (DHE) located on 2H.1, 5H.3 and 7H were detected, explaining rather large percentages of days to heading variation at the five trials (Table 5.6). The QTL located on 2H.1, between *PpdH1* and SNP 11_21015, was significant at three sites (Z09, Z10 and F10) but not at the two Lleida trials. This QTL explained 26.4, 8.6 and 26.4% of days to heading variation at Z09, Z10 and F10 respectively, with the Plaisant allele associated with earlier heading. At the two QTLs located on 5H.3 (at *VrnH1*) and 7H (between SNPs 11_10327 and 11_20074), the Plaisant allele was consistently associated with later heading at all trials although only the latter was a main effect as the larger effect at F10 resulted in the former being detected as a scaling effect QTL × environment interaction. Three of the four QTLs detected for time to maturity were in the same regions as the three DHE QTLs, with an additional QTL detected on 5H.2. As for DHE, the Plaisant allele at the locus in the region of *PpdH1* was associated with earliness at F10 but the character was not measured at the Zaragoza sites so it was only significant at the one out of three sites and its lack of effect at the Lleida sites may have affected its exact positioning on 2H.1. The QTL at *VrnH1* was the most significant for maturity, accounting for over 25% of the phenotypic variation at each site. Whilst the Plaisant allele increased maturity, as would be expected from its effect from DHE, the effect at F10 was much greater than at the Zaragoza sites so, like the DHE QTL, it was detected as a scaling effect QTL × Environment interaction. The QTL on 5H.2 was located between SNPs 11_10578 and 11_20850 with the Plaisant allele increasing maturity as a consistent main effect across all three sites. As for DHE, the QTL on 7H was a main effect with the Plaisant allele increasing maturity.

Five QTLs were detected for plant height, between *PpdH1* and SNP 11_21015 and at SNP 11_11505 on 2H.1, at SNP 11_10379 on 4H.1, between SNPs 11_20936 and 11_10954 on 6H and at SNP 11_20200 on 7H. The QTL on 7H was a main effect with the Plaisant allele contributing a consistent increase in plant height at all trials. The QTL on 6H was the most significant with the Plaisant allele increasing height at all four Spanish sites and accounting for over 13% of the phenotypic variation at any one but no significant effects were found at F10. The Plaisant allele at the second QTL on 2H.1 was also associated with a significant increase in height but only at Z09. On the contrary, the Orria allele significantly increased plant height at the other two QTL,

being significant at F10 for the first QTL on 2H.1 and at L10 and F10 for the QTL on 4H.1.

Three QTLs were detected for TGW located on 4H.1, 4H.2 and 6H, explaining 25, 19, 24 and 16% of the phenotypic variance for the character at L09, L10, Z09 and Z10, respectively. The QTL on 4H.1 was co-located with the plant height QTL at SNP 11_10379 and was a main effect with a consistent reduction associated with the Plaisant allele. Plaisant alleles at the other two QTL, at SNP 11_10610 on 4H.2 and at SNPs 11_20892 and 11_21469 on 6H were associated with significant increases in TGW in 2009 for the former and at all sites except Z09 for the latter. Six QTLs were detected for HEC. The one located on 2H.1 between SNPs 11_11430 and 11_10818, the same interval in which we found a yield QTL, was a main effect with the Plaisant allele increasing the character. The other five QTL were all QTL × Environment interactions and significant at three of the four sites. They were located on: 1H between SNPs 11_20267 and 11_20921, 2H.2 at SNP 11_21440, 3H at SNP 11_21362, 5H.1 between SNPs 11_20010 and 11_21065, and 7H between SNPs 11_20074 and 11_11014. The Plaisant allele at all but the QTL on 2H.2 and 7Hwas associated with increases in the character. The 2H.2 QTL was a cross-over interaction with the Plaisant allele significantly increasing HEC at L09 but decreasing it at L10 whereas Plaisant alleles at the 7H QTL significantly decreased the character at all sites except Z09. Grain width and area were only estimated at two trials, Z09 and Z10, with three and two QTLs detected, respectively. All but a grain width QTL on 5H.2 at SNP 11_20441 were detected as consistent main effects at the two sites. A QTL for both characters was detected at SNP 11_10379 on 4H.1, where we also detected a QTL for TGW, and, as for TGW, the Plaisant allele decreased each character. The other QTL for grain width was located between *PpdH1* and SNP 11_20105 on 2H.1 and the other grain area QTL was located on 1H between SNPs 11_20550 and 11_20267. The Plaisant allele associated with an increase for the former but a decrease for the latter.

Powdery mildew infection was estimated at Fiorenzuola d'Arda (F10), as there was an attack severe enough to reveal genotypic differences. The most significant QTL was located at SNP 11_10924 on 4H.1, where the Plaisant allele was the more resistant. The Orria allele was the more resistant at the other two QTL, which were located at SNP 11_10383 on 2H.2 and between SNPs 11_10056 and 11_10576 on 7H.

5.4 Discussion

Despite the narrow genetic base progeny from the cross Orria × Plaisant have proved remarkably high yielding in the Spanish National Barley Breeding Program with cultivars like Cierzo already commercialised. This study was therefore carried out to identify the favourable quantitative trait loci from each parent that have been recombined in the successful progeny. Unravelling the genetic factors underlying the agronomic advantages of this material for Mediterranean conditions will help optimize future breeding strategies to improve the chances of producing elite cultivars

The vernalization gene *VrnH1* was co-located with QTL on chromosome 5H.3 for grain yield, days to heading, and days to maturity in this population, with the Orria allele conferring earliness and significantly higher yield at three sites but significantly lower yield at Z10. Growing conditions were better at the two Lleida locations (L09 and L10), as manifested by higher grain yields and plant height of the parents and the population. Also, heading occurred earlier in Lleida than at Zaragoza, especially in 2009, even though the Lleida trial was sown later that season. This was caused by the warmer conditions experienced at the Lleida locations throughout the two seasons (Fig. 5.1). Consequently, the accumulation of growing degree days occurred faster at the Lleida (L) than at the Zaragoza (Z) sites. A significant delay in heading will reduce the grain filling period in Mediterranean environments where summer temperatures become excessive so the QTL effects detected for grain yield, days to heading and maturity are as we would expect for all sites apart from the Zaragoza ones, especially Z10. The delay in days to heading at Z10 was less marked than at the other sites and that difference coupled with greater late season moisture availability and/or a delay in the onset of high summer temperatures may have enabled the later heading types with the Plaisant allele to make use of a greater vegetative biomass and produce a higher yield.

Wang et al. (2010) reported an effect of *VrnH1* on grain yield in an advanced backcross study of a *Hordeum spontaneum* × elite spring barley population, although they did not detect an effect upon heading date. Sameri and Komatsuda (2007) also detected an effect in the region of *VrnH1* on grain number per plant and kernel weight with opposing effects of alleles from the parents, Azumamugi and Kanto Nakate Gold, but they did not assess heading date. No effect of *VrnH1* on grain yield was found in a study carried out in similar Mediterranean environments with the spring × winter population Beka × Mogador (Cuesta-Marcos et al. 2009), nor was it found to have any

significant effect on days to heading from an autumn sowing (Cuesta-Marcos et al. 2008a). Comadran et al. (2011) found significant QTL × Environment interaction for SNPs closely linked to *VrnH1* and *VrnH2* in a genome wide association study of yield for a diverse panel of barley genotypes that had been trialled over a number of different Mediterranean environments. Furthermore, Francia et al. (2011) reported significant effects of the developmental genes *VrnH1*, *VrnH2*, *PpdH2* and *Eam6* on grain yield both as main factors and in interactions with the environment from a study of the Nure × Tremois mapping population trialled at a number of Mediterranean environments. This study found that whilst *PpdH2* and *Eam6* explained a large proportion of the main genotypic effect, *VrnH1* explained the largest proportion of G × E interaction (17.6%) so our findings show considerable consistency with previous reports.

The QTL for grain yield on linkage group 2H.1 at SNP 11_11430, unlinked to *PpdH1*, seems to be in the area of a QTL hotspot for barley and is in the same region as SNP 11_10818, which we detected as a main effect QTL for hectolitre weight. Comadran et al. (2011) found a QTL for grain yield and days to heading on this chromosome at SNP 11_10191, less than 1 cM distant (Close et al. 2009; Muñoz-Amatriaín et al. 2011). Both Comadran et al. (2011) and Wang et al. (2012) identified a heading date QTL in this region in two different association panels. This region was reported by Borrás-Gelonch et al. (2012) as having a very large effect on days to heading and on the duration of developmental phases of barley and highlighted by Cuesta-Marcos et al. (2008a,b) as the one having the main earliness QTL for Mediterranean environments, co-locating with the gene *Eam6*. As we did not detect any associations with heading date in this region, it is possible that there may be more than one linked locus with differential effects at this region. Indeed, the region is centromeric so we can expect a number of linked genes in the region and the exact balance will largely depend upon parental origins as recombination will be restricted. The grain yield QTL, on 1H in the region of SNP 11_10725, does not appear to have been reported in elite barley crosses before although several authors have reported a grain yield QTL on 1H, in the vicinity of Bmac090 (Li et al. 2005; Bauer et al. 2009), from studies of H. spontaneum introgressions. Bmac090 is located 3cM away from SNP 11_10725 (WTB Thomas, unpublished data) so we could have detected a similar effect. Recently, Fisk et al. (2013) reported a frost tolerance QTL in the same region, in crosses NB3437f/OR71 and NB713/OR71, both involving at least one facultative parent. The closest marker to

the QTL was 11_10764 which is located in our map just 0.8 cM away from the marker closest to our yield QTL, 11_10275, so they both may be pointing at the same gene. This possibility is confirmed by the fact that there is a good agreement between the average temperatures of the Spanish environments for the first two months of the crop and the sign of the effects observed: at the coldest year, 2009 (4.7 ºC, average of December and January), the Plaisant allele at 11_10275 offered a yield increase (significant at Z09). In 2010, which was warmer (5.4 ºC), there were negative effects of the Plaisant alleles (significant at L10). This pattern, however, was broken by the the late sowing at F10 in which, if the yield QTL was actually a frost tolerance QTL, we would expect a negative effect of the Plaisant allele that did not occur. If the two QTLs are the same, there may be some interaction with other genes that masked its effect in this late sowing.

QTL for grain yield, flowering time and maturity were identified in a similar position on chromosome 7H. The closest marker to the QTL was SNP 11_10327, with the Orria allele associated with higher yield, earlier flowering and maturity. Notably, the effect for flowering time at this SNP was the only one for this character that we detected as a main effect. The effect for maturity at SNP 11_10327 was also detected as a main effect, although the trait was not measured at Z09 or Z10. The SNP's effect on grain yield was, however, detected as a QTL × Environment interaction but, whilst the effects detected at the Zaragoza sites contrasted to those at the other three, neither were significant. SNP 11_10327 is 5cM proximal to SNPs 12_30983, 12_30894 and 12_30895 (Close et al. 2009), which are all located in the developmental gene *VrnH3*. Wang et al. (2010) identified an effect of *VrnH3* on grain yield, and Ponce-Molina et al. (2012) detected a QTL for flowering date at the locus. Whilst the parents of our population differ for the promoter of *VrnH3* (unpublished data), the confidence intervals for the three QTL that we detected do not extend beyond SNP 11_10838, which is still proximal to the *VrnH3* SNPs (Close et al. 2009). It therefore appears very unlikely that the QTL that we have detected in this region of 7H reflects allelic differences at *VrnH3*. It is, however, noticeable that the confidence interval for the grain yield QTL overlaps with that for the hectolitre weight QTL that we detected in the region of SNP 11_20074. Here, the Orria allele also increases hectolitre weight so there would be considerable agronomic benefit to selecting for Orria alleles in this region for Mediterranean barley.

A large effect QTL for days to heading in the region of *PpdH1* on chromosome 2H has been found recurrently in several studies (von Korff et al. 2006; Li et al. 2005; Li et al. 2006; Bauer et al. 2009; Wang et al. 2010; Pasam et al. 2012). The QTL found in this study reinforces the importance of this locus for the control of flowering time in Mediterranean conditions, although it did not have a noticeable effect on grain yield, contrary to our findings for *VrnH1*. Laurie et al. (1994) reported a pleiotropic effect of *PpdH1* on plant height and yield components. Similar results were reported by other authors (von Korff et al. 2006; Bauer et al. 2009; Wang et al. 2010). In all cases, the later allele was associated with increases in plant height, as we have seen in the present study. The effect of *PpdH1* is, however, more marked under longer day lengths than those experienced in the current study so, whilst we also found that the later allele resulted in an increase in plant height, it is not surprising that we did not find any co-location of yield QTL.

The QTL for plant height on 6H is associated with SNP 11_10954, with the Orria allele reducing plant height. This marker is 1 cM proximal to the SSR marker Bmag0009 (WTB Thomas, unpublished data), which is associated with a plant height QTL in the Tadmor × ER/APM population (Teulat et al. 2001) and also overlaps with a QTL hotspot, including plant height, detected in the Tankard × Livet population (Rajasekaran et al. 2004), so there may be a general growth QTL still segregating in elite gene pools as well as landrace material in this region. The QTL for plant height in the region of SNP 11_20200 on 7H is in a similar position to a QTL for this trait found in the region of Bmag0516 by Rajasekaran et al. (2004) in the Tankard × Livet population. Bmag0516 is located just proximal to SNP 11_11219 (WTB Thomas, unpublished data) and thus is in a similar position to SNP 11_20200. Varshney et al. (2012) identified an association with plant height with the DArT marker bPb-2379, which mapped in the same position as SNP 11_20200 in the OWB mapping population (http://wheat.pw.usda.gov/ggpages/maps/OWB/, reported by Szűcs et al. 2009).The beta-glucan synthesis gene CslF6 (Burton et al. 2008) is located within 1cM of SNP 11_11219 and it is possible that the polymorphism that have and are being reported in this region are due to the persistence of high beta-glucan lines in non-malting barley types, which is linked to other genes of agronomic importance.

The most significant QTL for thousand grain weight was detected in the region of SNP 11_10379 on 4H.1. The QTL was detected as a main effect with Orria alleles

increasing the character, apparently through an increase in grain width and area as QTL for these characters were also found to be associated with SNP 11_10379. The SNP was also associated with a plant height QTL, with Orria alleles producing a significant increase in the character at L10 and F10. Using the maps of Muñoz-Amatriain et al. (2011) and Szűcs et al. (2009) and comparing locations of bin markers, we conclude that the QTLs associated with SNP 11_10379 are located in the same region as the thousand grain weight QTLs detected in Igri × Danilo (Backes et al. 1995) and Vogelsanger Gold × Tysofte Prentice (Kjaer and Jensen 1996). Thousand grain weight QTL reported by Li et al. (2006) and Baum et al. (2003) together with a height QTL detected in the region of HVM3 in Derkado × B83-12/21/5 (Chloupek et al. 2006), which is also in the same bin, add further support to our conclusion.

Another QTL for thousand grain weight was detected in the region of SNP 11_10610, which co-segregates with SNPs in the vernalisation gene *VrnH2* (12_30889 and 12_30892; Muñoz-Amatriain et al. 2011). This effect most probably reflects minor differences in vernalisation requirement affecting grain fill, although it has not manifested itself in changes in grain width or area. QTL for thousand grain weight have also been reported in the area of *VrnH2* (Teulat et al. 2001; Bauer et al. 2009).

It is noticeable that all the powdery mildew QTL are independent of the agronomic QTL. The most significant, in the region of SNP 11_20924 on 4H is in the same region as Bmag0353 and the bin marker bBE54A (Szűcs et al. 2009; Varshney et al. 2007). This would place the powdery mildew resistance QTL in the same region as the major resistance *Mlg*. Plaisant carries the resistant allele at the QTL but has only been reported as carrying the *Mlra* resistance gene (www.cprad.scri.ac.uk) so it is more likely that the effect that we have detected is the result of a minor gene rather than *Mlg*. Similarly, SNP 11_10383 maps between cnx1 and Zeo1 on 2H.2, which would place it in the same region as the major resistance gene *MlLa*. Neither Orria nor Plaisant are, however, likely to carry this gene and it is likely that the resistant allele carried by Orria again represents a minor gene. The confidence interval of the resistant QTL allele carried by Orria at SNP 11_10576 overlaps with those of the heading date and height QTL detected at the adjacent marker SNP 11_10327 and it is highly likely that shorter and earlier alleles of Orria render it less susceptible to powdery mildew. We therefore conclude that this most probably represents and escape mechanism rather than a true resistance effect.

Our data and those other studies indicate that grain yield under Mediterranean conditions depends to a remarkable extent on phenology, but also that not all phenology genes affect grain yield to the same extent or in the same manner. This effect of phenology on grain yield was already recognized in classical studies, although the genetic underpinnings were not fully understood at the moment. For instance, van Oosterom et al. (1993) already stated clearly that "development pattern has a marked effect on yield response across environments". This seems to be the case in this study, and in the others referenced in previous paragraphs, although the main genes determining genotypic effects and G × E interaction responses vary according to the genes segregating in each set of plant materials and the prevalent environmental conditions.

The clustering of traits in the principal component analysis gives an indication of their genetic control. The tighter distribution of points for plant height, days to heading and thousand grain weight suggests that they have higher heritability and/or are under simpler genetic control. The scattering of grain yield points over two quadrants, on the other hand, suggests a shift in the relationships among traits across trials. Grain yield was influenced by different sets of traits at different trials, probably as a result of a distinct reaction to diverse environmental conditions. This situation was confirmed by the fact that none of the four grain yield QTLs behaved consistently across environments. This is not unexpected under our conditions. Varshney et al. (2012), found a similar pattern in a recent association study with barley in the Mediterranean region, and attributed this fact to the differences in environmental conditions across sites triggering different genetic pathways, and to the strong conditioning of yield by earliness. Comadran et al. (2008), in an independent association study, found 43 QTLs for grain yield across 27 field trials across seven Mediterranean countries, but few were detected at several trials, and 22 were detected at only one trial. It is remarkable that the grain yield at autumn-sown trials in Lleida (L09 and L10) cluster close to F10, a March sowing in Italy, for which not much vernalization potential was expected, and not to the Zaragoza trials, which were located only 140 km apart. This indicates that the range of conditions that may be encountered in autumn sowings in Northern Spain can be remarkably wide in terms of vernalizing temperatures.

An important distortion of segregation for a number of loci was found in this population. Distortion of segregation in regions harbouring flowering time genes is

commonly observed in populations developed or multiplied under natural conditions. This seems to have occurred in linkage group 5H.3, due to selection at the *VrnH1* region. It may have occurred as well in the development of the population Nure × Tremois (Francia et al. 2011). In that population, two of the QTL for heading date were located in the regions of *Eam6* and *PpdH2* (Francia et al. 2004), and the frequencies of the markers used to tag these genes indicate a possible selection during the development of the population, with probabilities of 0.003 and 0.00006, respectively, according to a chi-squared test (own calculations based on supplementary data provided by the authors). Ponce-Molina et al. (2012) detected a strong selection towards the spring *VrnH1* allele, which induced a small vernalization requirement in the population SBCC145 × Beatrix (vs the alternative allele, which induced a higher vernalization requirement). This population was multiplied in a greenhouse, without any vernalization provided. Similarly, in Orria × Plaisant, we observed selection for the *VrnH1* allele inducing a lesser vernalization requirement. Orria has a unique *VrnH1* allele with reduced vernalization requirement. The first intron is similar to the HvVRN1-4 allele of Hemming et al. (2009) but it contains an additional 7 bp deletion within it (GenBank accession DQ492705). Under controlled conditions it behaves like the Spanish landrace SBCC058 (Casao et al. 2011, unpublished results). The RIL population was developed in Lleida and, therefore, its rather warm temperatures may have shifted the population towards an over-representation of the Orria allele at *VrnH1*, resulting in the distorted segregation observed in the linkage group 5H.3. During the advancement of the generations, occasionally some lines were discarded because they produced almost no seed, most probably because they had the Plaisant allele at *VrnH1* and, during warmer seasons, failed to flower normally.

Use of the QTL for MAS

The use of molecular markers can greatly increase selection efficiency, if the traits targeted are not severely affected by G × E interaction. Three of the four QTL detected in this study for grain yield show clear cross-over interactions and the remaining one was not clearly a scaling effect. It is therefore not evident which would be the best allele to select and a risk analysis would be necessary to identify the most appropriate allele. For instance, for the effect associated with *VrnH1* on 5H.3, it would be best to select for the Orria allele in environments where significant frost events are

unlikely to occur but it would be preferable to select for the Plaisant allele in environments where frost is more likely.

Reducing plant height is one of the goals of the current Spanish barley breeding program. Of the 5 QTLs found for plant height in this study, four showed interaction with the environment, but they were all scaling effects with no evidence of significant cross-over interactions. Thus, although two of the QTL were significant in just one environment and might not be such good targets for MAS, consistent selection for the shorter allele at any one of the five would be feasible. Favorable (short) alleles were derived from both parents, explaining the large transgressive segregation found for this trait (Table 5.3).

Considering the heading date QTL, appropriate selection strategies for *VrnH1* have been described above and, as Orria contributes the "early" allele consistently for the QTL on 7H, it can be used to adjust the growth cycle as necessary. The QTL at *PpdH1* appeared only at the Z and F trials. This is consistent with the well-proven effect of this gene under long photoperiod. Plaisant contributes the "early" allele at this locus, provided the plants are grown under long days. At both L trials, heading occurred too soon in the year for *PpdH1* to have any effect but it occurred later in the other three trials so that *PpdH1* had an effect on the growth cycle. We consider that the sensitive (Plaisant) allele should always be incorporated into winter cultivars for the Mediterranean area as it provides an insurance mechanism to induce flowering before temperatures rise too much in the season, which should also be built into the risk analysis strategy outlined above. The adaptive mechanism provided by photoperiod response has already been identified as one of the main forces driving the latitudinal spread of barley landraces in Europe with the sensitive *PpdH1* allele restricted to lower latitudes (Lister et al. 2009).

A possible antagonistic effect exists for the QTL in the region of SNP 11_10379 on 4H.1 as the Plaisant allele decreased plant height but also decreased grain weight and width. Selection for the Orria allele would appear to be the best strategy as the relative effect on grain weight is greater than that on plant height. Furthermore, the increase in plant height could be offset by selection at other plant height QTL, although some might be associated with undesirable effects on other characters not measured in this study. For instance, selection for the Orria allele at SNP 11_20200 would reduce height but, as

noted above, it should be verified that this might not affect grain beta-glucan content if breeding for the malting market.

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Chapter 6

Genomic regions affected by selection in a barley breeding program

6.1 Introduction

The use of molecular markers has become an important tool for genetic analysis and crop improvement (Rae et al. 2007; Varshney et al. 2007b). They are most commonly used for the exploration of genetic diversity, for the identification of genomic regions influencing traits of interest and for the selection of desirable phenotypes through the use of populations designed specifically for that purpose (Stuber et al. 1992; Mather 2002). But molecular markers can also be used to analyze existing populations and derive conclusions about the selective forces that have shaped their genomes.

Among other causes, the distortion of expected allele frequencies can be the result of selecting forces acting on particular genes (Falconer and Mackay 1996). When the segregation distortion is caused by differential viability of alleles, these loci themselves are of interest because they may help to understand the mechanism of selection (Zhan and Xu 2011). The analysis of distortion of segregation can thus be used to identify specific target regions for selection or loci closely linked to a distorted marker (Grini et al. 1999). This approach has been used extensively to analyze natural populations of organisms (Linhart and Grant 1996), but can also be attempted to analyze the outcome of breeding programs. Selection, either natural or artificial, over generations increases the frequencies of favourable alleles for the fitness of the organisms and, at the same time decreases the frequencies of less favourable alleles, therefore resulting in shifts in allele frequencies at the population level (Allard 1996; Danquah and Barrett 2002; Wisser et al. 2011).

In fact, it has been proposed that the evolution of allele frequencies of molecular markers during the selection process can be used to identify specific regions of the genome related to the trait(s) under selection (Wisser et al. 2008). The important alleles are enriched by selection and detectable by the analysis of allelic frequency shifts. This approach has been named “selection mapping”. Historically, there have been a number of studies conducted on the principle that phenotypic change can be explained by significant changes in allele frequencies between generations, at loci governing important characters due to selection. Classical studies of this kind in barley were carried out by Allard and collaborators (Jain and Allard 1960; Allard and Jain 1962; Allard et al. 1972; Clegg et al. 1972, 1978; Kahler et al. 1975; Allard 1988), but also by Hockett et al. (1983) and Charlesworth and Charlesworth (1998). In other cereal species, selection mapping has been used as a tool to monitor recurrent selection, as in

oat (De Koeyer et al. 2001), and maize (Stuber and Moll 1972; Labate et al. 1999 and Coque and Gallais 2006).

This study is a retrospective analysis of an elite cross from the Spanish National Barley Breeding Program. This cross was one of the most successful crosses in the breeding program and has resulted in an extremely productive progeny in the program. It has produced a large number of advanced lines, and has been a successful source of new cultivars in recent years. The lines from this cross will be investigated at two points in the program, before and after undergoing selection, through the analysis of allelic frequencies with molecular markers. The objective of this study was to search for genomic regions that may present selection footprints as a consequence of the breeding process, indicating possible targets for performing marker assisted selection in this and related crosses.

6.2 Materials and methods

The cross between cultivars Orria and Plaisant was done three times in the program, each at a different institute and year, with different direction of crossing: 93Z074 (made in Zaragoza in 1993, as Plaisant × Orria), 96V738 (made in Valladolid in 1996, as Orria × Plaisant) and 97L058 (made in Lleida in 1997, as Orria × Plaisant). Orria is a six-row winter cultivar with a mild vernalization requirement; it needs just around two weeks of cold temperatures to be fully induced towards heading and it is very productive across most regions in Spain. Plaisant is also six-row winter cultivar but it is a typical winter type that needs a considerable vernalization time to achieve timely induction of flowering and it is less productive than Orria.

The lines from these crosses will be investigated at two points in the program, before (F2) and after undergoing selection (F8). In the F2, the plants have not experienced conscious selection, whereas in the F8, the lines have undergone selection for several agronomic traits through five generations (from F3 to F7). The material was stored in an uneven way, and only one of the original F2s (Zaragoza, 1993) was available for analysis. A sample of 102 plants from this F2 and 41 of 45 advanced lines that reached the F8 generation in the breeding program (total number for the three crosses) were analyzed. Seed and DNA for 4 of the lines were lost.

The 102 individuals of the 93Z074 F2 population were genotyped using 28 polymorphic microsatellite markers (simple sequence repeats, SSRs), chosen randomly for neutral regions distributed throughout all the genome to study the population, and

two markers of flowering time genes, *VrnH1* and *PpdH1*. The F8 lines were genotyped using different markers: SSRs, SNPs and flowering genes (Table 6.1). Not all F8 lines were genotyped with the whole set of markers. There were some gaps due to different causes. Indeed, 39 lines had been previously genotyped with 32 random SSR markers in the framework of the breeding program and the data were incorporated to this study. The rest of the markers could only be analysed on lines for which either DNA or seed was still available in 2012. For the purpose of this study, 35 extant lines were characterized for 12 SNPs marking QTL regions derived from a previous study of a recombinant inbred line population (RILs) derived from cross 97L058, presented in Chapter 5. These last markers were chosen to represent regions that harboured important QTL for agronomic traits that were targeted in the breeding program, mainly grain yield, days to heading, plant height and kernel weight. When possible, the SNPs original markers were converted to gel-based markers. The closest markers to the QTLs, for which the conversion was possible, are listed in Annex 6.1, and the primers used are listed in Annex 6.2. In addition, markers for 3 flowering genes highly relevant for adaptation of barley to Mediterranean regions were genotyped in the F8 lines: *VrnH1*, *VrnH3* and *PpdH1*.

Table 6.1. Description of the genotypes and markers under study.

	F2 Population	F8 Population
Genotypes	102	41
All Markers	30	47
SSRs	28	32*
SNPs	-	12
Flowering genes	2	3
GBS-SNPs	-	936

* 28 markers in common among the two generations

In summary, the data for the F8 lines consists of SSR previously done in the breeding program, and new marker information generated specifically for this study. The analysis of these new markers was possible because either the seed or the DNA of the F8 lines had been preserved. Further work was done for the set of 31 F8 lines for which seed was preserved, by using the genotype-by-sequencing system (GBS, Poland and Rife 2012) provided by the company Diversity Arrays Technology (Kilian et al. 2012).

Segregation distortion was examined for allelic frequencies at the F2 and the F8, by testing deviations from Mendelian expectations without selection, using the Chi-square test provided by Microsoft Office Excel, for each marker, as recommended by Zhan and Xu (2011). For the GBS data, given the high number of tests performed at the single marker level, protection against false positives due to multiple testing was achieved calculating the false discovery rate (Storey and Tibshirani 2003) for the distribution of Chi-square single marker probabilities, as implemented in Genstat 14 (VSN International 2011).

The GBS sequences containing the SNP markers were assigned a location in the barley physical map (IBSC 2012), by using BLASTN (Altschul et al. 1990) against the datasets available at ftp://ftpmips.helmholtz-muenchen.de/plants/barley/public_data/ (updated 01-08-2012). Thresholds of 95% coverage and similarity of 98% for each 69 bp sequence produced by GBS were imposed to declare positive matches.

6.3 Results

Selection history of Orria and Plaisant crosses

The barley breeding program is carried out in four Spanish provinces following a strict pedigree scheme. Lines are derived from single heads of different plants at the F2, and advanced using a head-to-row system up to the F10. Early generation testing takes place from F3 up to F5, independently at each province. The F6 is the first generation of joint testing, in which the lines selected at the four provinces are merged together in joint trials. Selection in the first generations up to F5 focuses mainly on morphological and highly heritable traits (plant and spike appearance, height, flowering, healthy condition), and grain yield tests across locations start at F6.

The proportion of lines derived from the three crosses Orria×Plaisant increased in the advanced generations, and particularly after F6. In the first part of the program, up to F6, the relative frequencies of the lines from crosses between Orria and Plaisant tripled, but in the second part they almost increased fivefold (Table 6.2).

Attending to the grain yield expressed as percentage of the common check cultivars present at each trial, it was also clear that the averages of relative grain yield of the lines derived from these crosses were higher overall than the average of all lines derived from other crosses (Table 6.3). The reduction of relative yield observed for the F8 trials of the last cross (97L058) was due to the replacement of two of the checks used up to that moment (from a total of four) by two better cultivars. Therefore, the figure for this generation cannot be compared on par with the others.

Table 6.2. Selection history of the lines derived from three crosses between parents Orria and Plaisant in the Spanish barley breeding program.

	Cross 93Z074			Cross 96V738			Cross 97L058			Overall
Generation	**Year**	**Generation size**	**Number of lines 93Z074**	**Year**	**Generation size**	**Number of lines 96V738**	**Year**	**Generation size**	**Number of lines 97L058**	**percentage of O×P lines**
F3*	**1996**	20082	396	**1999**	13002	300	**2000**	4873	144	2.2
F4*	**1997**	1200	55	**2000**	286	12	**2001**	1201	75	5.3
F5*	**1998**	305	15	**2001**	149	12	**2002**	300	23	6.6
F6**	**99-00**	453	28	**02-03**	162	36	**03-04**	683	16	6.2
F7**	**00-01**	120	24	**03-04**	60	27	**2004**	120	9	20.0
F8**	**01-02**	53	15	**2004**	31	22	**2005**	64	8	30.4

* generations including lines from only one site (Zaragoza), ** joint program, with lines from all program sites

Table 6.3. Average of relative grain yield (expressed as percentage of common check cultivars) of the lines derived from the three crosses of Orria and Plaisant, compared to the overall average of all lines derived from other crosses evaluated in the same trials.

Cross	**Generation**	**Year**	**All lines n.**	**All lines relative grain yield**	**O×P lines n.**	**O×P lines reaching F8, relative grain yield**	**O×P lines not reaching F8, relative grain yield**
93Z074	F6	1999	453	95.5	28	118.7	106.5
	F7	2000	121	105.7	24	117.1	96.2
	F8	2001	53	103.9	15	107.0	
96V738	F6	2002	402	97.4	36	113.6	99.7
	F7	2003	80	110.1	27	114.3	109.7
	F8	2004	32	111.0	22	116.9	
97L058	F6	2003	842	97.8	16	112.3	112.6
	F7	2004	120	109.4	9	125.0	
	F8	2005	64	94.0	8	89.9	

Random SSR genotypic frequencies in the F2

A total of thirty polymorphic markers (microsatellites and flowering time genes) covering all chromosomes of barley was used to assess if there was any distortion of allelic frequencies before the beginning of line development in the breeding program.

The expected allelic ratio at any generation should be 1:1, if no selection or drift occurred. The expected genotypic ratio in the F2 should be 1:2:1. The observed ratios were tested for deviation from their expected values with a chi-square goodness-of-fit test ($P < 0.05$) for each marker. The allelic frequencies for the 30 markers in the F2 derived from the cross of 93Z074 did not depart from expected Mendelian allelic frequencies in 27 markers. Just three markers (10%) showed significant segregation distortion. Bmag0211 and Bmac0032, on 1H, showed an excess of Plaisant homozygotes and a deficit of Orria homozygotes, whereas one marker on 6H presented a reduced number of heterozygotes and a high number of Orria homozygotes (Table 6.4).

Random SSR allelic frequencies in the F8

The F8 genotypes showed an increased level of distorted frequencies. Out of the 32 polymorphic SSR evaluated, 12 SSRs showed distorted segregation. Therefore, 37% of the random loci in the F8 lines showed allelic frequencies significantly departing from the expected 1:1. Among these 12 loci, 11 were skewed towards Orria and just 1 was skewed towards Plaisant (Bmag0378, on 2H, Table 6.5).

Markers from genes and QTL regions

To test the genetic constitution of the F8 lines in the regions containing QTL, identified in the study of the RIL population (Chapter 5), BOPA1 SNP markers were converted into PCR-derived markers (Annex 6.1) and assayed in these plants. To do this, the position of some BOPA1 SNP markers used in Orria × Plaisant was compared to that found in the SNP consensus map of Muñoz-Amatriaín et al. (2011), to select new BOPA2 markers from the same regions. Targeted regions had QTL for yield (heading) QTL on 1H (2 markers), 2H (3 markers), 5H (1 marker) and 7H (4 markers) and plant height on 4H (2 markers). Other SSR or gene-specific markers were added on 7H to cover a wider region around the grain yield QTL. Regarding the plant height QTL identified on 6H, the SSR marker Bmag0009 was selected since it maps in the same position than the identified BOPA1 marker (11_10954) in the consensus map.

Regarding the markers from the QTL regions, out of 12 SNPs, 4 showed distorted segregation (Table 6.5). No distortion of frequencies was found for markers from the regions containing grain yield QTL on 1H or 7H. On the 2H region containing a grain yield QTL, a slight preference for the Orria allele was detected. A clear distortion was apparent for markers derived from the region containing a plant height QTL on 4H. Regarding the flowering time genes, *VrnH3* and *PpdH1* did not show segregation distortion, but *VrnH1* did. Actually, among the loci that showed segregation distortion in the F8, *VrnH1*, and its neighboring SNP 11_21241, showed the most extreme distortion. Out of 35 genotypes tested, 33 genotypes (94.3%) had the *VrnH1* allele of Orria and just 2 genotypes (5.7%) had *vrnH1* from Plaisant, whereas in the F2 population, the genotypes did not show any significant segregation at this gene.

Table 6.4. SSR markers used in the F2 population, number of genotypes at each marker locus, and X^2 probability calculated for the observed allelic frequencies (probability of being originated from random assortment of alleles in absence of selection).

			Genotypic frequencies			Allelic frequencies		
	Chr.	**cM***	Orria	Plaisant	Heter.	Orria	Plaisant	**X^2**
Bmac0399	1H	28.9	23	12	67	113	91	0.1
Bmag0211	1H	60.4	13	37	52	78	126	<0.01
HvM20	1H	66.3	15	20	63	93	103	0.5
Bmac0032	1H	73.7	14	36	52	80	124	<0.01
WMC1E8	1H	131.9	26	21	55	107	97	0.5
HvM36	2H	31.0	26	26	50	102	102	1.0
PpdH1	2H	25.1	30	25	47	107	97	0.5
Bmac0132	2H	67.0	22	31	49	93	111	0.2
Bmag0378	2H	76.1	25	28	49	99	105	0.7
HvM54	2H	122.4	28	28	46	102	102	1.0
Bmag0006	3H	50.1	19	23	59	97	105	0.6
Bmag0136	3H	52.1	19	23	60	98	106	0.6
Bmag0225	3H	75.5	21	30	51	93	111	0.2
Bmag0013	3H	113.7	28	17	53	109	87	0.1
Hv13GEIII¥	3H	130.0	28	22	49	105	93	0.4
HvM40	4H	22.4	24	26	52	100	104	0.8
Bmag0384	4H	57.5	26	27	47	99	101	0.9
HVM03	4H	58.3	25	27	50	100	104	0.8
Bmag0353	4H	65.0	26	29	47	99	105	0.7
EBmac0701	4H	96.2	29	26	46	104	98	0.7
HvM67	4H	120.5	28	25	49	105	99	0.7
HvBAMY	4H	133.3	36	23	41	113	87	0.1
VRNH1	5H	131.1	35	27	38	108	92	0.3
Bmag0173	6H	57.8	28	26	48	104	100	0.8
Bmag0009	6H	62.2	22	32	46	90	110	0.2
EBmac0806	6H	75.5	44	27	31	119	85	<0.05
Bmag0206	7H	15.3	18	19	65	101	103	0.9
GBM1116	7H	50.6	30	36	35	95	107	0.4
Bmag0120	7H	97.0	24	30	47	95	107	0.4
Bmac0156	7H	136.4	27	24	51	105	99	0.7

*cM is the position of the markers in the consensus map from Varshney et al (2007a) except *PpdH1* and *VRNH1*, whose locations are taken from Muñoz-Amatriain et al. (2011).

¥ Hv13GEIII was located approximately at 130 cM, 21 cM left of HvM70 in Silvar et al. (2010); HvM70 maps at 150.6 cM according to Varshney et al. (2007a).

Table 6.5. SSR, SNP markers and flowering time genes evaluated in F8 lines. Genotypic and allelic frequencies, and X^2 probability for the observed allelic frequencies (probability of being originated from random assortment of alleles in absence of selection).

			Genotypic frequencies			Allelic frequencies		
Marker	**Chr.**	**cM***	Orria[ψ]	Plaisant	Heter.	Orria	Plaisant	**$X^{2\dagger}$**
			Random markers					
Bmac0399	1H	28.9	18	19	1	37	39	0,87
Bmag0211	1H	60.4	21	16	-	42	32	0,46
HvM20	1H	66.3	18	20	-	36	40	0,69
Bmac0032	1H	73.7	16	21	1	33	43	0,46
WMC1E8	1H	131.9	25	12	2	52	26	<0.05
HvM36	2H	31.0	19	19	1	39	39	0,94
Bmac0132	2H	67.0	28	11	-	56	22	<0.01
Bmag0378	2H	76.1	12	27	-	24	54	<0.05
HvM54	2H	122.4	23	16	-	46	32	0,26
Bmag0006	3H	50.1	35	4	-	70	8	<0.01
Bmag0136	3H	52.1	35	4	-	70	8	<0.01
Bmag0225	3H	75.5	17	22	-	34	44	0,42
Bmag0013	3H	113.7	26	13	-	52	26	<0.05
Hv13GEIII[¥]	3H	130.0	29	10	-	58	20	<0.01
HvM40	4H	22.4	24	13	2	50	28	0,05
Bmag0384	4H	57.5	26	13	-	52	26	<0.05
HvM03	4H	58.3	29	10	-	58	20	<0.01
Bmag0353	4H	65.0	26	12	1	53	25	<0.05
EBmac0701	4H	96.2	24	14	-	48	28	0,13
HvM67	4H	120.5	20	17	1	41	35	0,63
HvBAMY	4H	133.3	20	18	-	40	36	0,75
Bmag0337	5H	45.0	18	20	-	36	40	0,69
HvLEU	5H	51.3	18	21	-	36	42	0,63
Bmag0173	6H	57.8	29	10	-	58	20	<0.01
Bmag0009	6H	62.2	24	15	-	52	30	0,09
EBmac0806	6H	75.5	28	10	-	56	20	<0.01
Bmag0206	7H	15.3	19	20	-	38	40	0,87
scssr07970[¥]	7H	27.0	17	18	-	34	36	0,87
GBM1116	7H	50.6	21	14	-	42	28	0,24
HvSS1[¥]	7H	59.0	19	20	-	38	40	0,87
Bmag0120	7H	97.0	17	21	-	34	42	0,47
Bmac0156	7H	136.4	22	15	-	44	30	0,29
			Flowering time genes					
PpdH1	2H	25.1	18	17	-	36	34	0.87
VRNH1	5H	131.1	33	2	-	66	4	<0.001
VRNH3	7H	31.1	18	17	-	36	34	0.87

Table 6.5. (continued)

			Genotypic frequencies			Allelic frequencies		
Marker	**Chr.**	**cM***	Orria[ψ]	Plaisant	Heter.	Orria	Plaisant	**$X^{2\dagger}$**
Markers related to QTL in Chapter 5								
11_10764	1H	34.1	16	19	-	32	38	0,61
11_20514	1H	35.7	16	19	-	32	38	0,61
11_20690	2H	77.8	23	12	-	46	24	0,06
11_20667	2H	82.9	24	11	-	48	22	<0.05
12_31394	2H	84.8	22	13	-	44	26	0,13
11_10379	4H	57.7	26	9	-	52	18	<0.01
11_10480	4H	57.7	26	9	-	52	18	<0.01
11_21241	5H	130.5	33	2	-	66	4	<0.01
12_10696	7H	47.6	18	17	-	36	34	0,87
12_10959	7H	52.0	20	15	-	40	30	0,40
12_30880	7H	54.5	21	14	-	42	28	0,24
11_10346	7H	55.1	23	12	-	46	24	0,06

*Position in cM of the SSR markers according to Varshney et al. (2007a) and of flowering genes *PpdH1, VRNH1* and *VRNH3* according to Muñoz-Amatriain et al. (2011)

¥ the position of these markers was estimated after comparison with the map by Silvar et al. (2010)

ψ39 F8 lines were evaluated with SSR markers, whereas 35 were tested with flowering time genes and SNP markers related to QTL.

† X^2 actually calculated for the allelic frequencies multiplied by 0.5, as the probability for one allele at a specific locus is almost completely conditioned by the other allele, in genotypes close to total homozigosity.

Genotype-by sequencing of the F8 lines

Seed or DNA of only 31 lines had been kept from the original 45. This subset of 31 F8 lines has been genotyped with the system of genotype-by-sequencing (GBS). Plaisant and Orria were polymorphic for 1177 SNPs and 2438 presence-absence events. A certain proportion of the SNPs was heterozygous for one of the parents, and was not used for further calculations. We used the information for 936 SNPs with less than 10% missing data for further analysis, as they provide enough coverage to search for selection footprints. These markers were scored in 31 F8 lines. Data for GBS PAV were not as complete as for SNPs, with a larger frequency of missing data and were not used for this analysis. The polymorphic SNPs presented allelic frequencies biased towards one of the parents in 264 cases (28.5%), 241 towards Orria (26.0%) and 23 towards Plaisant (2.5%). The Shapiro-Wilk test for the distribution of allelic frequencies (using the percentage of Orria alleles per marker), in classes of 5% increments, was calculated using Genstat 14 (VSN International, 2011). The test indicated that the distribution of

values for the 936 markers did not follow a normal distribution, with P<0.001. The distribution was skewed to the left, with many more alleles from the parent Orria and less from Plaisant (Fig. 6.1) than would be expected for a normal distribution of genes without selection (represented by the shaded area in Fig. 6.1).

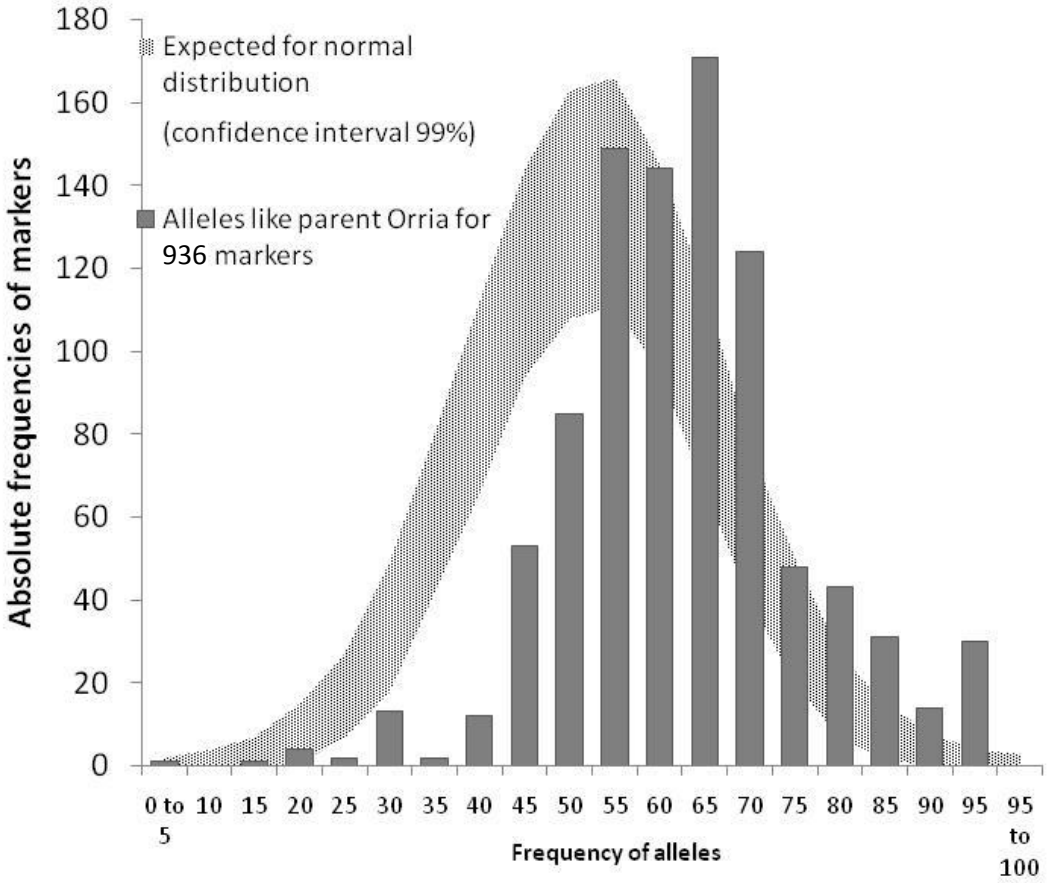

Figure 6.1. Frequencies of Orria alleles using 936 SNP markers with a genotype-by-sequencing system (bars). The shaded area represents the 99% percentile of frequencies that can be expected after sampling 936 markers that had not undergone selection (i.e., with 50:50 frequencies), with a standard deviation equal to the one derived from the distribution of frequencies observed. The percentile was calculated after running 1000 simulations.

Using the barley genome physical map (IBSC 2012) the position of the 936 SNPs was identified. The clones representing 419 SNPs matched either to a single locus or to multiple loci with a single map position, as they belonged to the same physical map contig. Another 345 SNP clones matched with multiple genomic locations with different map positions (Table 6.6). There were 172 GBS clones without any match to the reference sequence. In total, we found 1234 loci in the physical map for 764 SNPs.

The barley reference genome offers both genetic distance (cM) and base pair count (bp) on the 7 chromosomes. Using these data we analyzed the distribution of the allelic frequencies of markers over the recombination and physical distances (Figs. 6.2 and 6.3, respectively). The false discovery rate (FDR) procedure used to control for multiple testing indicated that absolute frequencies of above 71.5% or less than 30% for the Orria alleles were below the significance threshold chosen of $q<0.05$. The results of these figures also showed that there was significant segregation distortion in F8 lines in all chromosomes.

Table 6.6. Number of GBS SNPs matched with unique or multiple genomic locations in the barley physical map.

Chr.	Clones	
	Unique match	Multiple matches
1H	44	53
2H	67	47
3H	72	51
4H	50	58
5H	57	34
6H	46	29
7H	83	73
Total	419	345 (815)
No match	172	

An exam of Figs. 6.2 and 6.3 reveals visible patterns that can be interpreted as selection sweeps or selection QTL. The profiles are not completely clean, as the localization of the SNPs on the physical map carries some uncertainty. This is particularly true for the SNPs with multiple hits to the physical map. When we split these genome scans of frequencies in two, one for the SNPs with unique positions in the genome, and another for SNPs with multiple hits, the first ones offers cleaner profiles, although the overall picture of both is very similar (Annexes 6.3 and 6.4).

Many SNPs identified by GBS departed from 1:1 segregation, revealing some "selection footprints" on each chromosome. We chose to declare a selection QTL when there were at least two markers exceeding the thresholds, and the profile of the surrounding regions clearly hinted at the presence of a peak. A total of 11 regions were identified following this criterion, indicated in the Figs. 6.2 and 6.3. Most of these peaks were rather narrow, either considering physical or recombination distances. There was a remarkable exception at the QTL on 3H, possibly at a centromeric position, which

spanned over more than half of the chromosome. Plaisant alleles were favored on regions of chromosomes 1H, 4H and 7H, whereas Orria alleles were more abundant and showed conspicuous selection peaks on the rest. To relate the profiles identified in the F8 lines, and the results of the QTL analysis of a RIL population from this same cross, common markers were positioned *in silico* on the barley physical map (Table 6.7). These markers were chosen to tag QTL positions, or to match the regions of skewed allelic frequencies found with SSRs with the regions found with GBS SNPs.

Table 6.7. Markers or flowering genes chosen to relate the QTL or segregation distortion identified in the RIL population with GBS markers positioned in the barley physical map. Markers were positioned *in silico* in the barley physical map. Numbering indicates tags included for these markers in Figs. 6.2 and 6.3.

Chr.	Marker	QTL	Favorable allele	cM$^{\Psi}$	no. on Figs.	Other
Markers related to QTL in the RIL population						
1H	11_10275	Yield	O/P	42.77	1	
2H	*PpdH1*	Heading	P	22.17	2	
	11_20690	Yield	O/P	60.44	3	
	11_20667	Yield	O/P	67.35	4	
	12_31394	Yield	O/P	68.55	5	
4H	11_10379	Height	P	52.19	9	
5H	*VrnH1*	Yield , Heading	O	126.13	12	
6H	11_10954	Height	O	52.30	13	
7H	*VrnH3*	Yield	O	37.61	14	
	GBM1116	Yield	O	43.84	15	
	12_10959	Yield	O	53.19	16	
	11_10346	Yield	O	54.82	17	
Other markers						
3H	11_20866*	-		50.50	6	Distorted region around Bmag0006
	12_20591*	-		51.49	7	
	11_20801*	-		53.65	8	
4H	11_20765$^{¥}$	-		83.46	10	Distorted frequencies in RIL population
	11_11398$^{¥}$	-		87.70	11	

* Bmag0006 is close to ABG396 on the map of Varshney et al. (2007); these SNPs are close to ABG396 on the map of Muñoz-Amatriaín et al. (2011)
$^{¥}$ These SNP markers indicate a peak of distorted segregation in the RIL population (Fig 5.4).
$^{\Psi}$ position on the barley physical map (IBSC 2012)

The position of *VrnH1* on 5H matched a region showing an excess of Orria alleles at GBS SNPs, coincident with a clear deviation in the F8 lines for this allele and

also with a QTL identified in the RIL population for yield and heading date. Markers on 1H (11_10275), in the central part of 2H (11_20667) or in the long arm of 4H (11_10379), pointed to regions showing distorted segregation that were previously identified in the QTL analysis. On the other hand, no apparent distortion was detected in this analysis in the region of the 6H plant height QTL (11_10954) or the 7H yield QTL (GBM1116-11_10346). The results for chromosome 3H were surprising, with a large part of the chromosome, in the centromeric region, clearly deviated towards the Orria allele, as it had been detected above with SSR markers.

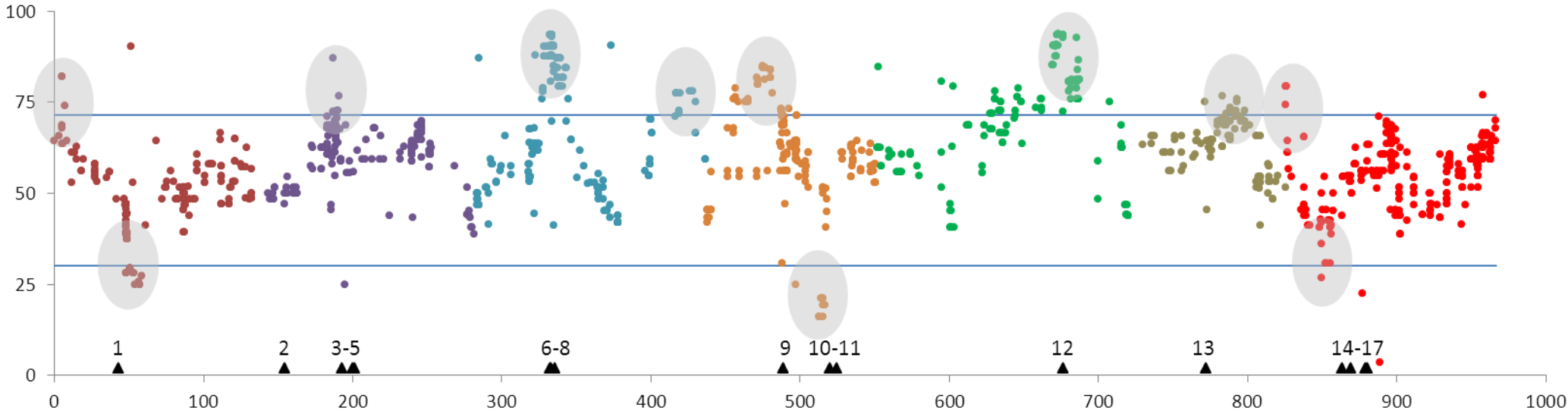

Figure 6.2. Segregation distortion in 31 F8 lines of the cross Orria × Plaisant using the genotype-by-sequencing system. Percentage of alleles from Orria vs cumulative centimorgan (cM) in the consensus map published by IBSC (2012). At the bottom of the graph, black triangles indicate the position of other known markers in the physical map, numbered according to Table 6.7. Shaded areas indicated the regions with selection QTL declared (see text).

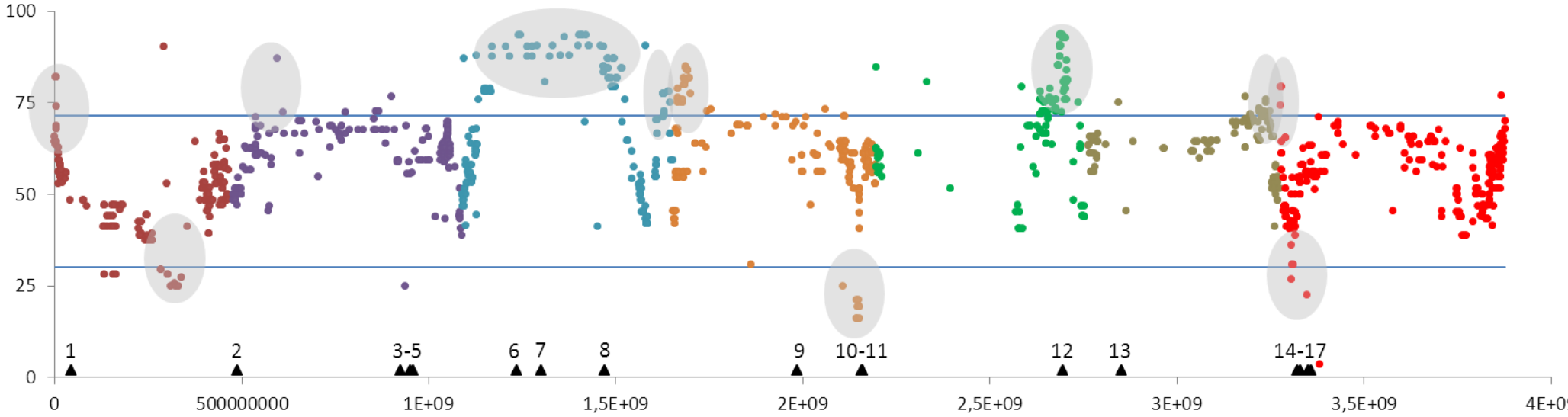

Figure 6.3. Segregation distortion in 31 F8 lines of the cross Orria × Plaisant using the genotype-by-sequencing system. Percentage of alleles from Orria vs cumulative base pair (bp) in the physical map published by IBSC (2012). At the bottom of the graph, black triangles indicate the position of other known markers in the physical map, numbered according to Table 6.7. Shaded areas indicated the regions with selection QTL declared (see text).

6.4 Discussion

In a breeding program, selection is carried out by phenotypic evaluation in different environments, selecting the best individuals according to several traits, and promoting them to the next generation. We expect that the outcome of this process will be an increase of the proportion of favourable alleles at loci important for adaptation to environmental conditions and predominant stresses as generations advance. Through the breeding program, the materials have an associated history selection. The relationship between the selection histories and the genomic regions affected can be investigated retrospectively through technologies of markers and genetic association.

We have studied the allelic frequencies of F2 plants and F8 lines derived from the cross of Orria × Plaisant, the two most elite parents of the Spanish National Public Barley Breeding Program. The individuals of the F2 have not experienced artificial selection, but the lines of F8 have already suffered selection through five generations (from F3 to F7), first for morphological and highly heritable traits (plant and spike appearance, height, flowering) from F3 to F5 and then for mainly grain yield in F6 and F7.

The proportion of lines from the crosses analyzed increased throughout the program as generations advanced, but the increase was particularly high after F6 (when selection for grain yield started), indicating that the advantage of these lines lied particularly on a high grain yield potential.

The F2 analyzed showed little evidence of allelic frequencies different from the expected 50:50. We have no reason to believe that the frequencies in the F2s of the other two crosses were different than in the one actually analyzed The comparison of allelic frequencies between the F2 and F8 of the same cross in the current study, on the other hand, revealed considerable differences between the generations, that are most likely the result of artificial selection.

In a similar study, Condón et al. (2008) used SSR markers to analyze changes in allelic diversity in a barley breeding program carried out between 1958 and 1998. They found evidence for a reduction in number of alleles at some markers. The authors hypothesized that it was the result of linkage of these markers with major loci for disease resistance or malting quality, and that were presumably under selection during the breeding process. Several authors have indicated changes in allelic frequencies, with a reduction in modern cultivars (Russell et al. 2000; Karakousis et al. 2003). Similarly,

Fu and Somers (2009) using wheat microsatellites reported that allelic reduction occurred in every part of the wheat genome as a consequence of breeding.

Only one of the F8 lines showed alleles inconsistent with the parents. This result is not unexpected. Actually, Sjakste et al. (2003) reported a much higher frequency of inconsistent alleles at a comparable study, 13.9%. The presence of off-type alleles could be explained by cross-pollination during some step of the breeding program. The result for this line was confirmed by two independent data sets, DArT and BOPA1-SNPs (data not shown).

Karakousis et al. (2003) revealed that using microsatellites, several SSRs assessed in F2 crosses showed distorted segregation, while others showed the expected 1:2:1 ratio. They explained this result as a consequence of preferential amplification of alleles, resulting in the inability to detect heterozygotes for some markers. We cannot be sure, but this same reason could explain the deviation from expectations observed in our study for EBmac0806.

Various studies of highly variable barley populations have reported changes in genotypic and allelic frequencies between generations, apparently as a result of selection for local adaptation. Clegg et al. (1978) studied the Composite Cross V (CCV) barley population (Suneson 1956). The F3 and all subsequent generations were grown from random samples taken from the harvest of the preceding generation and no conscious selection was practiced at any time. In that study they examined generations 5 to 10, 15 to 21, and 25 to 30, and found that the genetic composition of the population had changed substantially during the different generations. Saghai Maroof et al. (1994) found similarly dramatic allelic frequency shifts in barley Composite Cross II (CCII) after 53 generations.

Changes due to consciuous artificial selection have been reported in maize (Stuber et al. 1980, Romay et al. 2012) and oat (De Koeyer et al. 2001) when evaluating the outcome of recurrent selection programs.

What genomic regions were apparently selected during breeding? In Chapter 5 we used a population of recombinant inbred lines (RILs), derived from one of the crosses under study, 97L058, to identify favourable QTLs for grain yield and other agronomic traits. In this population, we found an important segregation distortion on chromosome 5H, at the *VrnH1* region. This distortion at *VrnH1* was towards the allele of the parent Orria. Also, QTLs for days to heading and maturity were detected at this same region. The hypothesis to explain this finding was already advanced in Chapter 5,

and was based on the different vernalization requirement induced by the *VrnH1* alleles of these two parents, reacting against different winter temperatures at the field trials, and resulting in a strong selection towards the Orria allele at this locus. In this study, we found further evidence of the strong selection pressure affecting this gene under Mediterranean conditions. The region containing *VrnH1* was clearly selected, against the Plaisant allele, during the breeding process. This allele was associated with lower grain yield, later heading and later maturity (Chapter 5), and we can assume that, besides affecting overall plant fitness, the selection put on these traits by the breeders favored the selection against this allele. Besides *VrnH1*, the highest distortion was detected for two markers on chromosome 4H, flanking a plant height QTL. In this case, there was preferential selection for the Orria allele. Although this allele was associated with taller plants, it was also associated with higher thousand grain weight, and wider grains with larger area. Regarding another plant height QTL on 6H, we used Bmag0009 that maps very close to the QTL peak. For this marker, we also detected distorted segregation with more F8 lines carrying the Orria allele, associated with shorter plants, again consistent with the selection pressure exercised during the breeding process.

The genotype-by-sequencing system produced a high number of markers, well spread throughout the genome. These markers provide a complete genetic profiling of a representative subset of the F8 lines. The results of GBS showed that over 90% of the loci with skewed frequencies were veered towards Orria. These results suggest a higher value of the Orria alleles in this cross. Deviation towards Orria is surprising and somewhat unexpected, except on the *VrnH1* region on 5H. Plaisant alleles were found preferentially selected in the long arm of 1H, at the end of 4H and in the short arm of 7H. Some of these regions were close but did not match the position of the QTL identified in a parallel study with a RIL population from this same cross (Chapter 5). The results for chromosome 3H are intriguing. Selection during the breeding process has led to the fixation of a large part of this chromosome from Orria, as it was suggested from the data for two SSR markers (Bmag0006 and Bmag0136) in the F8 lines. Based on the position of these markers in consensus maps (Varshney et al. 2007a; Aghnoum et al. 2010; Muñoz-Amatriaín et al. 2011), we identified several SNP that were located in that region on chromosome 3H, confirming that this was the same region in the GBS analysis. We can only speculate about possible reasons for the preferential selection of the Orria allele in this chromosome. Other authors have reported grain yield, lodging or height QTL in that region of 3H (Hayes et al. 1993; von Korff et al. 2008), but no QTL

was identified in that region in our QTL analysis of agronomic traits in the RIL population. Whatever is the reason behind this distortion; it must have taken place abruptly, in the early generations of the breeding program and led to a skewed distribution of the alleles. Indeed, no distorted segregation was detected on chromosome 3H in the RIL population. QTL for disease resistance, i.e. net blotch (Cakir et al. 2011), spot blotch (Roy et al. 2010) or scald (Li and Zhou 2011) were identified in other studies in that region of 3H, although we are not aware of a resistance for those diseases segregating in this population. Another possibility may relate with spike morphology (Chen et al. 2012) or head shattering (Larson et al. 1996), traits for which QTLs that have been located in that region of 3H. We do not know if there was any disease attact severe enough to justify the selection found at this region. On the other hand, we know that head shattering was heavily selected against during the early generations of the program. We can speculate that this trait, head shattering, is a more likely candidate underlying the 3H selection QTL, but further experimental proof is needed.

The comparison of the profiles of allelic frequencies across the genome for the advanced F8 lines and for the RIL population presented in Chapter 5 (with a different set of markers) delivered some surprising results. Two of the selection QTL with Plaisant as the favourable allele appeared to be in similar regions as the two regions with strongest allelic distortion in favour of the Plaisant alleles, on chromosomes 1H and 4H. The region on 1H is actually in the vicinity of *Fr-H3* (Fisk et al. 2013), a frost tolerant locus coincident with a GEI QTL locus in the RIL population (Chapter 5). Therefore, we have an additional proof of the relevance of this region in the breeding process. The region on 4H co-locates exactly with the region presenting heavy distortion of allelic frequencies in the RIL population (Chapter 5), as indicated by the location of markers introduced for comparison purposes in Figs. 6.2 and 6.3 (11_11398 and 11_20765). It is striking that two sets of materials with different selection histories present similar selection footprints. In the RIL population, lines are advanced almost without selection. If lines are discarded is because they do not survive at some generation. Therefore, only those traits that affect fitness severely can underly the selection observed in the RIL population. The fact that the same three regions on 1H, 4H and 5H appear as affected by artificial selection (but also by underlying natural selection) in the F8 lines, in the same sense in each case, confirms that they were not due to chance. Possibly, the fitness traits underlying these genomic regions acted in the

same direction in the two independent processes of material development, and their effects are so important as to be detected readily in each case.

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Chapter 7

General Discussion

Chapter 7: General Discussion

This thesis focuses on barley adaptation and improvement in Mediterranean conditions. The overall objectives were to test the efficiency of selection in a breeding program carried out in Mediterranean conditions and to find the most important factors responsible for the genetic progress in elite material in the program, to facilitate future selection. These aspects have been considered using both retrospective studies of materials generated during the activities of the Spanish National Barley Breeding Program, and the best elite material currently available in the program. Retrospective analysis of changes in genetic gain and phenotypic variance can be useful in designing strategies to manage genetic variation for target traits in breeding programs (Condón et al. 2009).

This section will focus on these issues, following approximately the structure of the chapters, but organized around the set of questions that were established from the beginning of this project, or that arose while working with the data. The first main question was:

1. Was breeding effective in developing better barley cultivars?

The short answer is yes, because the varieties that are being released pass the rigorous thresholds for the official registry, demonstrate good performance in independent trials, and are readily adopted by the industry. But the purpose of the first part of work is a scientific audit of the progress attained in the program. The Spanish National Barley Breeding Program aims to obtain barley varieties with adaptation to the main Spanish production regions, with stable yield, quality and tolerance to biotic and abiotic stresses. The program follows a strict pedigree system, and is carried out in a joint manner since 1995 by institutes located in four representative provinces. Up to this date, independent programs were carried out in each province. The joint program started as an attempt to scale up the breeding activities, to optimize the use of combined resources, to reduce redundancies, to address a target area covering the most representative barley growing regions of Spain, and to provide a larger set of environments for field tests, something particularly useful under Mediterranean conditions.

After approximately 20 years of joint program, there are enough plant materials with an associated history of selection to carry out an appraisal of its success. By

focusing on the materials developed over a representative period of time, it should be possible to assess the efficiency of selection and the progress in the advanced stages of the program across the locations used, to be sure that the program is achieving its goal of producing genotypes with high yield potential and stability across the whole targeted area (chapter 3).

Studying the progress attained at the advanced generations of the program over time indicated that the program is reaching its main goal of producing superior barley genotypes with high yield potential and stability suitable across Spanish barley growing regions. There was evident progress in the program, with increasing grain yield over the generations, even compared with increasingly better check cultivars, to the point that the most advanced lines surpassed the performance of the checks in the last generations. The effectiveness of selection was satisfactory across all provinces of the program at a majority of locations.

2. Was the breeding program equally effective for all regions?

Even after realizing the success of the program, this study detected two situations in which the progress was lower than in the rest, namely the limited progress attained at the advanced generations in locations V1 (Valladolid) and A1 (Albacete dry-land). The distinct features easily identifiable for these locations are productivity for A1, the lowest of the program (Table 3.3), and temperature for V1, the coldest location (actually, a group of locations) of the program (Fig. 3.2). Being at one extreme of the distribution of productivity probably causes that the overall genetic correlation of A1 with all other locations is the lowest. As a result, whatever genetic mechanisms favour grain yield at A1 are the less likely to be represented when selection is based in overall means. Productivity itself cannot be considered at the basis for the low progress in A1, as shown in chapter 3. 'Productivity' is a general term that comprises a wide array of features. We cannot be sure of which specific factors cause the reduction in productivity at A1 but, given the prevailing climate, reduced water availability should be among them.

We expect that the level of production found at A1 is not uncommon in Spain. The national productivity varied between 1500 up to 3700 $kg.ha^{-1}$ from 2000 to 2010 (data derived from Table 1.1), with an average of 2800 $kg.ha^{-1}$. The average yield of the advanced trials of the program is 3650 $kg.ha^{-1}$, thus slightly above national averages. Yield estimation on small plots, as is the case for the advanced trials, cannot be very

accurate, and we cannot rule out a slight over- (or under-) estimation of the yields calculated in the program. Also, many studies have identified a yield gap between experimental stations and farmer fields, usually due to better management of experimental fields (Lobell et al. 2009). Given these data, and even allowing for some uncertainty of the productivity levels found in the program, it looks that the productivity of location A1 must be representative of a rather large area of the Spanish barley cultivation regions. Therefore, specific activities towards finding materials better suited for the consistently dry areas of the country should be attempted.

Regarding location V1, the effect of winter temperature in the selection of plant materials in the breeding program is one of the main findings of this work, and it is dealt with more extensively later in this section. Regarding future prospects for breeding, it seems advisable to look for materials better suited for the coldest regions of the country, as V1.

Given the advantages of the structure of the current program (logistic, but also scientific, as wide adaptation has been achieved in the cultivars released), these goals could be implemented as a side line of the main program, rather than as a separate program.

3. Was GEI present in the breeding program?

A retrospective analysis also allows studying the amount of genotype-by-environment interaction (GEI) arising in the program (chapters 3 and 4). This interaction reduces genetic gains in breeding programs by reducing the heritability of traits selected. If the amount of GEI follows a geographic pattern, in our case genotype-by-province (considering the four provinces of the program), then decisions should be made about whether the program can be continued with the same geographic structure, using the same provinces and locations, or should it be changed. The results of this retrospective analysis indicate that the predictable parts of the GEI, as Genotype by Province interaction and Genotype by Location were not high, nor repeatable. Therefore the program can be continued with the same geographic structure, using the same provinces and locations.

But was there any other identifiable cause of GEI? To find answers to this question, a study was initiated using the best quality genotypic data available, and putting them in relation to environmental variables.

4. Were there any identifiable causes of GEI?

It is not easy to identify the causes underlying GEI. But, over the years, a good number of studies point at the relevance of different adaptation to temperature and rainfall as the main factors responsible for the occurrence of GEI in barley (Voltas et al. 2002, and references therein). Other causes that have been found in a few studies include soil conditions and reactions to diseases. Recently, a large study which classified barley trials from all over the world in three characteristic regions, found that GEI among the regions was mainly caused by variation in rainfall patterns, by disease incidence and, to a lesser extent, by reactions to temperature (Hernández-Segundo et al. 2009). Interestingly, locations from Spain were placed in two different regions, meaning that Spanish environments may be quite diverse. In chapter 4, we have found that GEI in Spanish conditions is, at least partially influenced by the reaction to winter temperatures.

The climate of the Spanish barley production regions tends to be warmer than the climate of more northerly European regions where winter barley is also grown. Therefore, we can expect to find some differences in the type of varieties that may be better suited to autumn sowings under the Mediterranean conditions. Furthermore, the conditions for barley production in Spain are variable, partially because vernalization conditions are also variable (Cuesta-Marcos et al. 2008, 2009). In our study we found confirming evidence for this, due to the variation in winter temperatures across trials. The cultivars reacted to these conditions depending on their genetic constitution regarding growth habit.

Throughout this study, the Spanish trials showed large GEI for grain yield and days to heading, and the patterns of GEI were influenced by barley growth habit. Particularly, we found significant interaction between the winter temperature of the trials and the *VrnH1* alleles for the traits; grain yield and days to heading. All these results indicate that the Spanish locations provide different vernalization potential. In chapter 4, we saw that the advanced lines with small vernalization requirement were less affected in the trials with intermediate and high temperature compared to the lines with strict winter *VrnH1,* which need to be exposed to a low temperature for a longer period.

The finding of a selection QTL at a region that co-locates with *Fr-H3* a recently proposed locus for frost tolerance (Fisk et al. 2013), on the short arm of chromosome

1H, suggests a role for this trait during the selection of the lines. Frost tolerance had been suspected as a factor of discrimination among genotypes under Spanish conditions, but has been rarely identified as a main factor at any of the selection trials. Frost tolerance is very difficult to assess at field trials. Results are often "inconclusive, due either to complete winterkill, or a lack of winterkill at any particular location" as stated by Limin and Fowler (1991). Also, recent studies establish clear connections between the vernalization and cold acclimation regulatory gene networks (Gáliba et al. 2009). In this study, we have established a relevant role of the interaction of vernalization with winter temperature on grain yield GEI. On the other hand, we have to keep in mind that the progress due to breeding was minimal at the coldest location of the program.

Given all these facts, at this moment there are enough hints to speculate about a possible role of frost tolerance on grain yield GEI in the cross of Orria and Plaisant and also in the entire program.

5. Which were the genetic foundations for the successful cross(es) of the breeding program?

The cross between Orria and Plaisant has proved to be one of the best crosses of the Spanish National Barley Breeding Program. This cross has resulted in a large number of lines reaching the final stages of the Spanish National Barley Breeding Program, and has been a source of successful new cultivars in recent years, characterized by a wide range of adaptation across the Spanish environments, already available in the market. Investigating the genetic factors that underlie the advantageous traits in this cross may facilitate implementation of marker assisted selection for barley breeding for Mediterranean conditions.

The main objective of studying the population derived from this cross was to identify favourable quantitative trait loci (QTL) for agronomic traits, and some important QTLs were found. But through this study we observed that a very important segregation distortion occurred in the region surrounding the *VrnH1* locus. Being a RIL population, multiplied with a head-to-row system since the F2, without selection, no genetic distortion was expected. The parents of this population differ in their vernalization needs. Orria is a facultative cultivar and has a very mild vernalization requirement. Plaisant is a winter cultivar with strict winter growth habit. This segregation distortion in the *VrnH1* region seems to have occurred due to unintentional

selection at a location with relatively warm winters, as explained in chapter 5. Most likely, what happened is that during the advancement of the generations, occasionally some lines were discarded because they produced almost no seed, probably because they had the Plaisant allele at *VrnH1* and, during warmer seasons, failed to flower normally.

To explore whether this segregation distortion in the *VrnH1* region has occurred due to selection, we investigated the lines derived from the crosses between Orria and Plaisant at two points in the program, before and after suffering selection in two generations; F2 and F8. The allelic frequencies in the F2 were consistent with little or no selection, but the lines in F8 have already suffered strong selection for yield, plant height and other agronomic traits through five generations (from F3 to F7), and their genotypes clearly reflect shifts in allelic frequencies, more concentrated at certain parts of the genome. We have found selective sweeps in this population, some with a clear explanation, but others just open to speculation.

The segregation distortion in the F8 population was marked, reflecting the differential effect of selective forces on regions of the genome, particularly at the *VrnH1* region, but also at other regions surrounding other fitness genes such as *Fr-H3*. This indicates that the changes in the allelic frequencies from F2 to F8 probably occurred due to selection for adaptation to the environment. This kind of change occurs for traits related to survival when local adaptation occurs at loci with strong phenotypic effects, resulting in strong selective sweeps (Le Corre and Kremer 2012). A similar occurrence of selective sweeps at the *VRN1* loci (the loci corresponding to *VrnH1* in wheat) was observed in wheat populations left to evolve under natural selection in different regions of France (Rhoné et al. 2008, 2010). In those studies, the *VRN1* alleles that resulted selected at each region were related to the prevailing climate, particularly regarding winter temperatures. In our case, the explanation would be that selection for the *VrnH1* allele from Orria occurred as it is better adapted and more suited to the Mediterranean regions with milder winters, as Lleida. This strong selection opens the possibility for performing marker assisted selection for this gene attending to the alleles of the parents and the characteristics of the target region, as was demonstrated recently by Casao et al. (2011).

Selective sweeps, or selection QTLs, as a result of breeding activities have been found in close crops like rice (Steele et al. 2004) or wheat (Raquin et al. 2008; Wang et

al. 2012), and may be a source of QTL for further investigation or to directly select candidate genomic regions for MAS.

Eleven selection QTLs were found, using rather strict criteria, when analyzing the distribution of allelic frequencies of the GBS results in the F8 lines, 8 with Orria as the donor of favourable alleles and 3 with Plaisant. On the other hand, some selection QTLs were found when analyzing the GBS results that were not the same as the QTLs detected in the RIL population, most notably the marked peak found at 3H. These QTLs should be due to selection for traits that were (consciously or not) considered in the breeding program and that have not been considered in the study in chapter 5. Some candidates for these traits are frost tolerance, disease tolerance and head shattering. Field winterkill was observed sporadically and, certainly, not every year, but it should be considered as a candidate trait attending at the co-location of *Fr-H3* with selection footprins in Orria by Plaisant in Chapters 5 and 6. Also, selection against diseases and against head shattering was very strict in early generations. Though disease attacks were not common, it may have been a relevant factor if its effect accumulated over the years. Actually, families were discarded if they showed any sign of disease and also if there was any hint of spikes falling to the ground late in the season. Either one of these traits may cause a selective sweep as strong as the ones observed on chromosomes 1H and 7H (towards Plaisant), or on 3H, towards Orria, for which no close QTL was found.

The QTLs found in chapters 5, through linkage mapping in a RIL population, and chapter 6, selection QTLs in advanced breeding lines, confirmed each other to a limited extent, with no certain matches, beyond that of *VrnH1*. The information from the selection QTLs may actually be more trustable than mapping based on phenotyping of the RILs. These selection QTLs are based on a long history of breeding, in three crosses, and are the summary of strong selection pressures over many years on large populations. Even though they are based on a limited number of lines (31 for the GBS data), they actually represent the result of selection among over 800 F2 plants, and against many other populations. So any strong evidence for a selection footprint must be the result of the selection forces acting during the breeding. Therefore, the regions identified as selection QTLs are very strong candidates for further study or for carrying out marker-based selection in the future.

6. **What future prospects can be derived from these results?**

The results presented in this thesis suggest several possible avenues to continue research or to use them in applied breeding. Some of them are:

1. Enrich the breeding program with materials destined to increase productions in the driest areas of Spain, as Albacete, and materials better suited for the coldest regions as Valladolid.
2. Increase the proportion of the crosses between winter and intermediate and spring and intermediate materials in the breeding program, to obtain materials that offer a wide range of vernalization requirements, and possibly cold tolerances, better suited to each region.
3. Trying to use genomic selection (GS) in the cross of Orria×Plaisant, and close crosses, for identifying and tagging the favorable alleles at regions indicated by the QTLs and selection QTLs. This information can be used following different strategies. One is to construct an ideal genome of the cross with favourable regions from Orria and Plaisant, then produce a large cross, and recover plants with genomes closest to the ideal one, using a high throughput SNP assay, to continue breeding within the remaining segregating regions. Another strategy could be to produce crosses of Orria×Plaisant parentage with materials of different origins, and use a high troughput SNP assay to screen for the favourable alleles at the selected regions, and select only the plants with segregation in other genomic regions, seeking better alleles not fixed in the Orria×Plaisant cross.
4. Extend GBS testing for all F8 lines in the breeding program, and searh for other possible selection QTLs. Use that information to identify the fixed favorable regions in the varieties which are often used as parents in the breeding program. Also search for crosses which present polymorphism in neutral or negative regions. Then proceed with early generation marker assisted selection with high-throughput SNP platforms to select for alternative alleles at these neutral or negative regions.
5. Investigate if the grain yield QTL on 1H is actually a frost tolerance QTL with appropriate experiments with the RIL population. Also, find out if the 3H selection QTL is a head shattering QTL.
6. Investigate if the allele *VrnH1-4* offers an agronomic advantage in different genetic backgrouns (for instance, in crosses with spring cultivars).

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Chapter 8

Conclusions

Chapter 8: Conclusions

1. The progress due to selection at the Spanish National Barley Breeding Program was confirmed. Grain yield, the main goal of the program, increased from F8 to F10, surpassing the level of the checks. We can conclude that the program is reaching its main goal of producing and identifying superior barley genotypes.
2. The effectiveness of selection was satisfactory across all four provinces, though differences were observed among particular locations. The selection was more successful at a majority of locations, and less in others, especially at V1 and A1.
3. The program can be continued with the same geographic structure, using the same provinces and locations, but attention should be paid to traits for specific adaptation to locations where it was less successful.
4. Selection gain at the last step (F9-F10) clearly decreased compared to the gain achieved at the previous step (F8-F9). The causes for this reduction should be investigated.
5. The genotype-by-environment interactions for grain yield and days to heading are influenced by growth habit under Spanish conditions. There is a relationship between genotype-by-environment interaction for grain yield and vernalization requirement. The variable winter temperatures occurring across the Spanish barley growing areas lead to differential responses of the genotypes according to their growth habit.
6. Only one consistent QTL for grain yield was found, on chromosome 7H. The rest of the QTL for grain yield presented interaction with the environment. In two cases, these QTL can be attributed to different reaction to frost (1H) and to vernalizing temperatures (5H).
7. Vernalization requirement, as a result of the allelic segregation at *VrnH1* is the major driver of genetic variation for grain yield in the cross of cultivars Orria and Plaisant. Segregation distortion and the strongest yield (and heading date) QTL in the RIL population, and the strongest selection QTL in the F8 lines coincide in the paramount relevance of this gene for this particular cross. Allelic variation at this gene is crucial to barley adaptation to Mediterranean conditions.

8. The retrospective analysis of materials from different generations of the breeding program is confirmed as a useful method to identify genomic regions affected by selection, or selection QTLs.
9. The causes of the occurrence of several selection QTLs have been found through comparative analysis with other studies and populations.
10. Some consistent QTLs for relevant agronomic traits and some strong selection QTLs have been found, and are proposed to carry out marker-assisted selection in the breeding program.

Annexes

Annex 4.1. Monthly average temperature for the testing locations during barley growing season in period 1 and period 2.

Trials	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.
03A1	10.6	8.7	5.5	6.1	11.1	13.2	17.9	25.6
04A1	10.8	7.0	7.9	8.2	9.1	11.8	14.8	23.6
05A1	9.3	6.6	4.9	4.9	10.7	13.8	19.5	24.5
06A1	8.7	5.9	4.6	6.8	11.8	15.4	19.6	22.8
07A1	12.0	6.7	6.3	9.8	9.8	12.3	17.4	22.5
09A1	8.2	6.1	5.5	7.9	11.3	12.5	19.3	24.4
10A1	12.5	6.9	5.7	7.2	9.4	14.1	16.1	21.6
03A2	10.6	8.7	5.5	6.1	11.1	13.2	17.9	25.6
04A2	10.8	7.0	7.9	8.2	9.1	11.8	14.8	23.6
05A2	9.3	6.6	4.9	4.9	10.7	13.8	19.5	24.5
06A2	8.7	5.9	4.6	6.8	11.8	15.4	19.6	22.8
07A2	12.0	6.7	6.3	9.8	9.8	12.3	17.4	22.5
08A2	9.2	6.8	8.1	9.2	11.1	14.3	16.3	21.4
09A2	8.2	6.1	5.5	7.9	11.3	12.5	19.3	24.4
10A2	12.5	6.9	5.7	7.2	9.4	14.1	16.1	21.6
02L1	6.0	-0.7	4.1	6.8	10.5	11.9	15.3	21.2
03L1	8.7	6.3	3.5	4.6	9.4	12.6	17.0	24.6
06L1	7.7	1.6	3.6	4.0	10.5	14.3	19.1	23.1
07L1	10.7	3.1	4.1	7.0	8.6	13.7	17.3	21.4
08L1	4.9	3.7	5.0	7.2	9.4	13.2	17.2	21.4
09L1	7.0	3.3	4.1	6.2	9.6	12.1	19.8	23.6
10L1	10.5	5.5	4.8	5.3	8.7	13.7	16.8	21.6
02L2	7.9	0.3	6.2	9.2	12.7	14.2	16.8	22.8
04L2	10.6	6.9	7.7	5.7	9.2	12.6	16.9	23.9
06L2	9.5	2.2	4.9	5.8	12.4	15.3	20.1	23.6
08L2	7.6	5.5	6.3	9.7	11.5	14.6	17.8	21.8
10L2	11.8	6.4	6.0	7.0	10.0	14.5	17.0	21.4
00L3	7.1	5.4	2.9	10.2	11.8	13.8	20.1	22.9
01L3	9.5	7.9	7.7	7.6	14.2	14.3	18.4	23.5
03L3	11.3	8.3	5.7	6.8	12.3	14.8	18.6	26.3
05L3	8.4	6.7	3.4	5.5	10.4	14.8	19.6	24.4
07L3	12.6	4.2	5.2	9.3	11.2	14.8	18.7	22.8
09L3	7.9	5.4	5.3	8.2	11.0	13.1	20.3	24.3
02V1	5.9	1.3	5.7	6.8	9.5	11.4	13.9	21.0
05V1	5.8	4.3	2.2	3.0	8.6	11.9	16.5	22.6
06V1	7.0	4.1	2.9	4.0	9.4	12.1	17.0	21.4
07V1	10.6	4.0	4.5	7.6	7.8	12.0	14.7	17.9
08V1	6.2	3.5	5.7	7.8	8.0	11.2	14.0	18.7
09V1	6.2	3.8	3.8	6.0	9.4	10.0	16.3	20.3
10V1	10.0	5.0	4.8	4.8	7.7	12.2	13.6	18.5

Annex 4.1. (continued)

Trials	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.
00V5	4.4	3.9	-0.2	6.9	6.5	7.3	13.9	18.3
02V5	4.0	0.2	4.5	5.4	7.2	9.2	11.4	18.8
03V5	7.6	5.8	2.4	3.3	9.2	9.2	13.3	20.8
05V5	4.1	2.7	1.2	0.7	8.0	9.4	14.1	20.0
06V5	5.4	2.7	1.6	2.2	7.3	10.0	15.1	19.7
07V5	9.5	2.5	3.3	5.6	5.7	9.1	12.5	15.9
08V5	4.5	2.9	4.7	7.0	6.0	8.9	12.0	16.8
09V5	4.1	2.1	2.8	3.7	7.8	8.1	14.4	19.3
00Z1	6.9	5.7	3.7	9.2	9.8	10.8	18.4	21.7
01Z1	8.6	7.7	6.9	7.2	12.3	11.9	16.3	21.6
02Z1	7.6	2.9	6.4	7.7	11.6	11.9	15.2	21.5
03Z1	10.5	8.0	5.0	4.7	10.6	12.3	15.6	24.2
04Z1	9.4	5.7	6.6	4.5	6.9	10.5	14.4	21.6
05Z1	7.5	6.1	3.2	3.4	9.5	12.3	17.0	23.4
06Z1	8.3	3.3	5.1	5.0	10.5	13.1	17.9	22.0
07Z1	12.1	4.0	5.8	8.3	8.3	13.5	15.6	19.2
10Z1	9.8	4.8	4.5	5.3	9.9	12.3	13.8	18.7
01Z2	8.9	8.1	6.8	7.7	12.9	13.6	17.6	22.7
03Z2	10.6	8.4	5.9	6.0	11.0	13.5	17.7	25.5
04Z2	9.8	6.6	7.4	4.9	8.1	11.6	16.4	23.5
07Z2	11.8	3.7	5.1	8.4	9.7	14.3	17.7	21.9
09Z2	7.9	5.2	4.4	7.1	10.4	12.5	19.0	23.3
10Z2	10.7	5.8	5.3	5.7	9.2	13.8	16.0	21.0

Annex 4.2. Monthly average rainfall for the testing locations during barley growing season in period 1 and period 2.

Trials	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.
03A1	39.7	28.4	19.4	55.6	23.2	43.8	41.1	9.4
04A1	20.1	14.3	3.9	36.6	64.7	85.5	71.0	9.8
05A1	3.1	26.4	0.3	17.0	12.8	25.0	0.9	11.5
06A1	27.6	9.7	43.9	12.7	9.2	35.2	29.6	21.8
07A1	66.5	4.4	12.2	18.1	49.4	78.4	23.8	13.0
09A1	24.5	31.9	45.6	15.2	61.5	24.4	14.6	14.7
10A1	4.3	120.2	89.2	64.6	61.9	41.4	31.9	38.5
03A2	39.7	28.4	19.4	55.6	23.2	43.8	41.1	9.4
04A2	20.1	14.3	3.9	36.6	64.7	85.5	71.0	9.8
05A2	3.1	26.4	0.3	17.0	12.8	25.0	0.9	11.5
06A2	27.6	9.7	43.9	12.7	9.2	35.2	29.6	21.8
07A2	66.5	4.4	12.2	18.1	49.4	78.4	23.8	13.0
08A2	5.1	6.8	10.5	22.8	12.8	27.0	98.7	134.5
09A2	24.5	31.9	45.6	15.2	61.5	24.4	14.6	14.7
10A2	4.3	120.2	89.2	64.6	61.9	41.4	31.9	38.5
02L1	49.0	7.0	20.0	10.0	37.0	89.0	63.0	50.0
03L1	47.0	44.0	26.0	103.0	49.0	24.0	6.8	4.0
06L1	52.0	15.0	60.0	7.0	7.0	47.1	13.0	14.0
07L1	13.0	11.0	9.0	6.0	28.0	152.0	26.0	23.0
08L1	12.0	0.0	22.0	13.0	4.0	88.0	167.0	52.0
09L1	43.0	41.0	42.0	31.0	44.0	124.0	7.0	35.0
10L1	8.0	68.0	63.0	32.0	60.0	20.5	49.0	68.5
02L2	49.7	13.8	22.9	4.3	17.0	38.1	44.4	44.7
04L2	52.3	17.4	5.3	50.6	36.6	70.0	53.5	5.8
06L2	57.5	8.0	33.6	3.5	7.0	6.8	4.2	3.6
08L2	7.7	2.0	17.8	14.0	2.3	40.0	112.7	60.8
10L2	3.7	43.1	73.3	25.7	34.3	25.5	36.8	91.7
00L3	31.7	2.7	1.6	0.0	39.2	46.0	30.8	54.1
01L3	47.1	35.5	20.4	3.9	24.0	77.2	38.8	7.7
03L3	24.5	18.1	16.9	70.2	28.9	27.2	60.0	32.5
07L3	7.2	16.1	11.5	11.6	26.4	68.2	25.7	8.1
05L3	2.3	29.1	2.6	8.2	10.1	2.9	49.6	9.7
09L3	23.1	27.9	28.1	19.7	33.5	104.6	3.6	28.5
02V1	4.6	5.5	45.4	11.3	32.7	38.0	38.7	8.6
05V1	39.2	16.9	2.7	8.9	13.2	38.5	16.0	6.4
06V1	49.2	22.5	40.2	43.3	32.8	59.3	9.8	72.6
07V1	82.9	16.1	17.5	44.0	17.5	63.0	90.0	66.6
08V1	51.1	8.3	40.0	38.7	5.6	83.2	162.7	40.5
09V1	25.6	59.9	35.1	20.1	3.0	21.3	23.9	36.8
10V1	23.5	108.9	66.7	55.2	47.9	64.7	25.4	41.5

Annex 4.2. (continued)

Trials	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.
00V5	1.2	35.6	13.9	0.0	27.7	63.0	87.0	4.6
02V5	0.8	4.2	39.6	8.8	37.8	33.7	60.9	17.6
03V5	67.8	35.6	67.3	39.0	34.2	78.3	22.0	27.1
05V5	23.9	14.2	1.2	20.9	20.9	32.1	14.3	8.4
06V5	47.4	27.1	33.0	28.9	28.0	24.6	10.2	33.7
07V5	41.4	17.9	14.0	56.5	12.6	57.8	87.4	106.3
08V5	41.0	7.3	19.3	25.5	13.2	65.7	99.6	43.0
09V5	15.6	46.0	44.8	13.8	5.8	31.4	13.0	23.0
00Z1	38.1	22.2	2.5	4.7	21.0	97.1	74.5	35.0
01Z1	80.2	59.6	53.0	6.0	48.0	24.5	10.0	4.2
02Z1	13.2	13.0	8.6	20.1	30.2	45.2	64.0	53.0
03Z1	35.5	60.9	75.4	60.5	41.3	28.8	33.1	15.1
04Z1	62.7	22.0	18.7	40.4	56.5	73.0	56.5	0.0
05Z1	15.5	41.1	3.5	12.4	5.2	50.5	61.6	25.1
06Z1	64.4	34.1	36.3	27.5	48.0	102.3	45.7	32.8
07Z1	25.6	9.8	18.9	53.8	99.1	128.5	20.2	9.6
10Z1	95.8	35.5	33.8	37.4	43.0	40.4	36.1	77.1
01Z2	70.0	38.4	40.6	2.5	37.1	5.1	61.4	3.7
03Z2	31.4	32.8	30.9	34.3	28.3	27.3	64.1	14.9
04Z2	55.5	16.7	7.0	37.5	40.4	39.6	38.2	0.9
07Z2	19.3	10.3	10.6	25.1	48.6	97.2	29.3	24.0
09Z2	39.2	48.5	22.8	10.7	20.4	62.6	14.9	11.3
10Z2	27.7	52.4	47.6	34.1	38.3	17.6	22.3	18.5

Annex 4.3. Productivity average of grain yield of the winter and spring genotypes in different trials, in the last three generations of the Spanish Barley Breeding Program in period 1 (2000-2004).

Trials	Alpha	Barberousse	Graphic	Zaida
00L3	6597	6911	8438	6807
00V3	6735	7268	6432	5591
00V5	7518	7896	7614	7899
00Z1	2844	2425	3292	2471
01L3	5548	6734	7236	6345
01Z1	3171	3659	4599	3449
01Z2	3197	2981	3222	2895
02L1	6291	5962	5503	5545
02L2	7990	7015	9011	6828
02V1	1824	1835	2241	2024
02V5	6846	7340	7019	6863
02Z1	2973	2560	3454	3103
03A1	3056	3108	3366	3362
03A2	6140	6119	6071	5055
03L1	3703	4145	3748	3990
03L3	7688	9457	9133	8566
03V5	3118	3266	3453	2683
03Z1	3331	3514	3472	2644
03Z2	5096	5415	5146	4842
04A1	5128	5132	5259	4488
04A2	4202	3879	4511	3883
04L2	7431	7107	8310	6268
04Z1	5782	6500	5292	4913
04Z2	3807	3936	3236	3204

Annex 4.4. Productivity average of grain yield of the winter and spring genotypes in different trials, in the last three generations of the Spanish Barley Breeding Program in period 2 (2005-2008).

Trials	Barberousse	Cierzo	Graphic	Hispanic
05A1	1542	1664	1742	1654
05A2	7387	7897	8311	8679
05L3	6660	6862	6363	6816
05L4	4721	5090	4142	3822
05V1	3118	3407	2850	3574
05V5	3308	3542	3140	4209
05Z1	4257	4749	4854	5480
06A1	3661	3896	3155	3637
06A2	5204	6271	5529	5959
06L1	3801	3719	3237	3304
06L2	7071	7086	7995	6640
06V1	2253	2306	2521	2581
06V5	2802	3361	2657	2785
06Z1	6172	6931	6864	6442
07A1	4557	6231	5378	5592
07A2	8895	10397	9758	9940
07L1	4902	6357	4613	4064
07L3	6701	7787	7482	6540
07L4	6571	6993	5301	5347
07V1	7428	7097	7233	7845
07V2	5849	6181	6586	6986
07V5	6705	7068	7621	6996
07Z1	8473	9819	10954	8703
07Z2	4925	5977	5435	5330
08A2	6152	7297	6564	7010
08L1	5297	5160	4926	3909
08L2	6369	8264	6116	6036
08L4	5270	5935	5574	4330
08V1	9284	8984	9322	8855
08V5	6785	8395	6238	6733
09A1	4179	5419	4892	5211
09A2	8375	9414	9635	9173
09L1	5112	5915	5239	4986
09L3	9109	8284	9054	8564
09V1	4398	4455	4328	4398
09V5	3427	3692	2871	3554
09Z2	3324	3527	3163	3439

Annex 4.4. (continued)

Trials	Barberousse	Cierzo	Graphic	Hispanic
10A1	4740	4833	4031	4108
10A2	7961	8285	6635	6458
10L1	4737	5395	4488	4422
10L2	8621	8735	9063	7930
10V1	7419	6547	6305	6432
10Z1	7095	7537	7526	7402
10Z2	3341	3285	2920	3702

Annex 4.5. Days to heading average of the winter and spring genotypes in different trials, in the last three generations of the Spanish Barley Breeding Program in period 1.

Trials	Alpha	Barberousse	Graphic	Zaida
00L3	114	111	115	112
00V5	120	123	129	123
01L3	104	106	102	101
01Z2	100	100	100	96
02L2	103	105	105	100
02V1	122	125	124	123
02V5	123	122	129	123
02Z1	117	120	121	117
03A1	118	115	119	116
03A2	134	132	135	132
03L1	114	115	115	113
03L3	105	105	105	104
03Z1	120	119	123	124
03Z2	109	110	107	103
04A1	116	116	116	118
04A2	108	110	113	112
04L2	109	110	104	103
04Z1	126	125	139	130
04Z2	125	125	127	125

Annex 4.6. Days to heading average of the winter and spring genotypes in different trials, in the last three generations of the Spanish Barley Breeding Program in period 2.

Trials	Barberousse	Cierzo	Graphic	Hispanic
05A1	121	121	123	123
05A2	118	122	124	119
05L3	106	107	105	104
05V1	120	121	124	120
05V5	122	124	124	119
05Z1	118	119	121	110
06A1	111	113	115	113
06A2	115	115	117	116
06L2	100	102	97	96
06V1	136	137	140	136
06V5	119	115	120	116
06Z1	115	118	122	112
07A1	125	126	127	125
07A2	128	127	129	127
07L1	111	110	104	101
07L3	106	105	103	97
07V1	127	130	129	125
07V5	114	124	120	113
07Z1	120	120	120	115
07Z2	122	121	123	116
08A2	123	123	126	120
08L1	118	122	123	117
08L2	115	118	118	114
08L4	127	128	133	127
08V1	123	126	127	120
08V5	112	116	115	112
09A1	131	130	124	116
09A2	120	120	122	116
09L1	114	118	119	111
09L3	106	109	107	104
09V1	117	120	119	114
09Z2	114	115	114	110
10A1	124	126	132	124
10A2	122	122	124	121
10L1	120	120	124	116
10L2	111	112	109	105
10V1	117	118	117	114
10Z1	118	121	121	117
10Z2	117	118	118	112

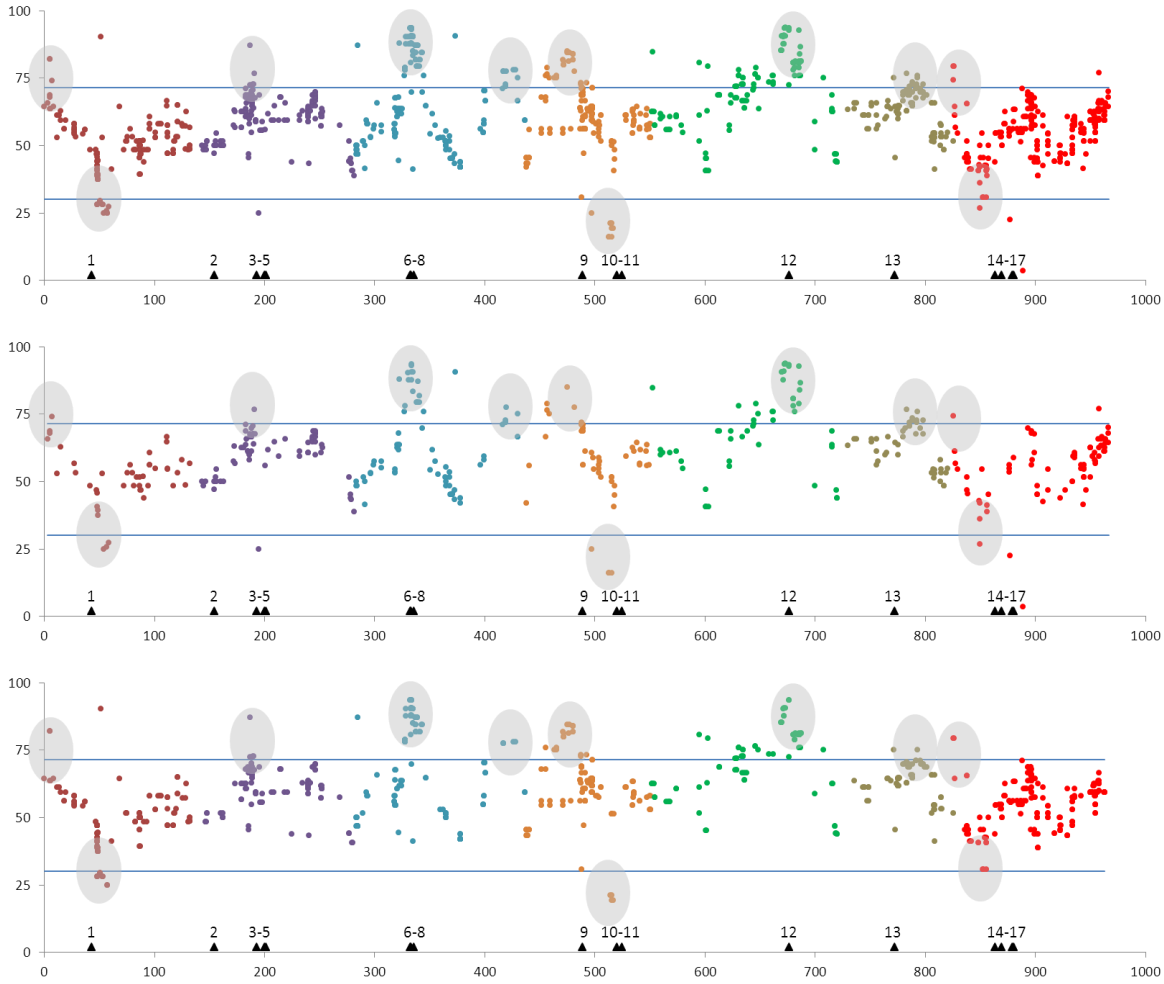
Annex 6.1. BOPA1 SNP markers and flowering time genes targeting the QTL regions for yield, heading date or plant height identified in the Orria x Plaisant RIL population. Closest marker to the QTL peak, tested markers in the F8 lines, position in the OxP map and in the consensus map of Muñoz-Amatriaín et al. (2011) are shown.

	QTL peak	closest marker	Tested markers	OxP	Consensus
YLD					
1H	44.6	11_10275	-	44.6	35.45
			11_10764	43.8	34.14
			11_20514	44.3	35.66
2H.1	54.1	11_11430	-	48.9	73.23
			11_20690	-	77.83
			11_20667	-	82.94
			12_31394	-	84.75
5H.3	14.8	*VrnH1*	*VrnH1*	14.7	131.13
			11_21241	14.8	130.46
7H	58.2	11_10327	-	52.6	35.93
			VrnH3	-	31.06
			GBM1116	-	-
			12_10696	-	47.63
			12_10959	-	52.04
			12_30880	-	54.49
			11_10346	68.9	55.14
DHE					
2H.1	5.9	*PpdH1*	*PpdH1*	0	25.1
PHE					
2H.1	33	11_11505	-	33	51.41
4H.1	62.5	11_10379	11_10379	62.5	57.68
			11_10480	62.7	57.68
6H	25.2	11_10954	-	27.1	58.72
			Bmag0009		58.72
7H	87.1	11_20200	-	87.1	81.4

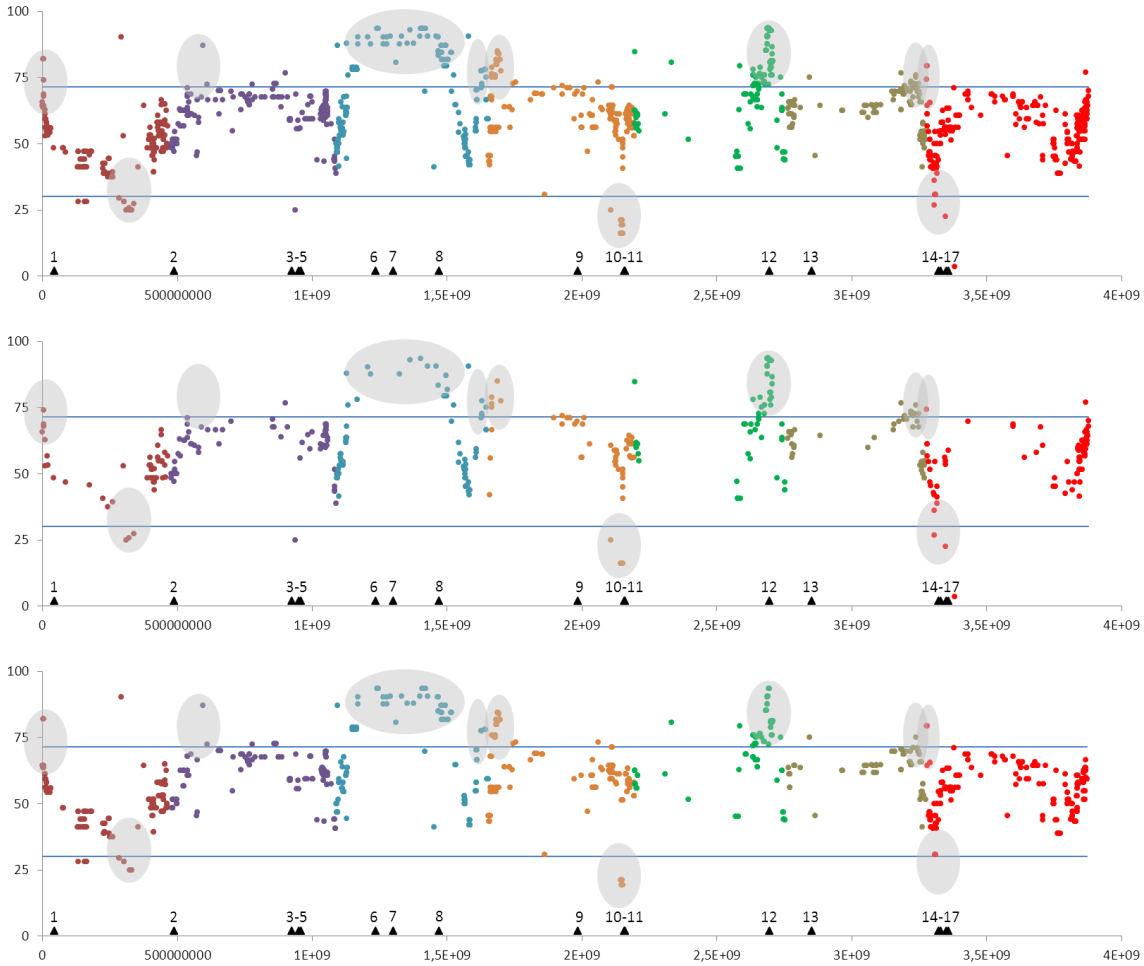
Annex 6.2. Primers used to amplify new SNP-derived markers.

Chr.	Marker	Forward primer	Reverse primer	Polymorphism
1H	11_10764	GTGCAGAGACGACACCAGAG	TTGGAAGGCATGGAAGAAAT	*Alu I*
1H	11_20514	ATGCTCATGACCCATGTTGA	ACGTGGATGTTCAACGCATA	*Hha I*
2H	11_20690	CAGATGAGAGTCCTGCACCA	TATCGGCAAAACAACCAACA	*Tsp45 I*
2H	11_20667	CTAAGGAGGGCCGGTTATCT	TAACGGCATCCCTCATCTTC	presence/absence
2H	12_31394	TTTCAGTCGGGACCAATCTC	ACGTCTGCCCACGTAATAGC	*Hpa II*
4H	11_10379	TAACCCGAAGCTGGTTTTTG	CTGCACGAAATGGATTGATG	*Hae III*
4H	11_10480	AGCGAGTGCTCAAGGAGAAG	CAGATGACCAGAACGCAAAA	presence/absence
5H	11_21241	AGGCTCGCTATTGGAAGGTT	TCAGCCTTGTCAGAAACACG	*Rsa I*
7H	12_10696	TGATGCTCTCAAGCTTCCAA	GTCAATTAGCGGCAGGAAAA	*Hpa II, HpyCH4 V*
7H	12_10959	GCTTCAGGAGTTCTGCATCC	CAGGCTGTTTGCAGAATGAA	*Fsp I*
7H	12_30880	TTCACAGCTGACCTGATTGC	GCTCCTCCCTATCCTTGGAC	*Rsa I*
7H	11_10346	CGAGACAACCAAGGAGAAGC	ACGCCACAACAATAGGCAAT	*Hha I*

Annex 6.3. Segregation distortion in 31 F8 lines of the cross Orria × Plaisant using the genotype-by-sequencing system. Percentage of alleles from Orria vs cumulative centimorgan (cM) in the consensus map published by IBSC (2012). At the bottom of each graph, black triangles indicate the position of other known markers in the physical map, numbered according to Table 6.7. Shaded areas indicated the regions with selection QTL. Top graph, all markers; intermediate graph, markers with unique position; bottom graph, markers with multiple positions in the physical map.



Annex 6.4. Segregation distortion in 31 F8 lines of the cross Orria × Plaisant using the genotype-by-sequencing system. Percentage of alleles from Orria vs cumulative base pair (bp) in the physical map published by IBSC (2012). At the bottom of each graph, black triangles indicate the position of other known markers in the physical map, numbered according to Table 6.7. Shaded areas indicated the regions with selection QTL. Top graph, all markers; intermediate graph, markers with unique position; bottom graph, markers with multiple positions in the physical map.



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Progress in the Spanish National Barley Breeding Program

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Abstract

The Spanish Barley Breeding Program is carried out by four public research organizations, located at the most representative barley growing regions of Spain. The aim of this study is to evaluate the program retrospectively, attending to: i) the progress achieved in grain yield, and ii) the extent and impact of genotype-by-environment interaction of grain yield. Grain yields and flowering dates of 349 advanced lines in generations F8, F9 and F10, plus checks, tested at 163 trials over 11 years were analized. The locations are in the provinces of Albacete, Lleida, Valladolid and Zaragoza. The data are highly unbalanced because the lines stayed at the program for a maximum of three years. Progress was estimated using relative grain yield and mixed models (REML) to homogenize the results among years and locations. There was evident progress in the program over the period studied, with increasing relative yields in each generation, and with advanced lines surpassing the checks in the last two generations, although the rate of progress was uneven across locations. The genetic gain was greater from F8 to F9 than from F9 to F10. The largest non-purely environmental component of variance was genotype-by-location-by-year, meaning that the genotype-by-location pattern was highly unpredictable. The relationship between yield and flowering time overall was weak in the locations under study at this advanced stage of the program. The program can be continued with the same structure, although measures should be taken to explore the causes of slower progress at certain locations.

Additional key words: genotype-by-environment interaction; *Hordeum vulgare*; pedigree selection.

Resumen

Progreso en el programa nacional español de mejora de cebada

El programa nacional de mejora de cebada se lleva a cabo por cuatro organismos públicos situados en las principales regiones productoras de cebada de España. El objetivo de este estudio es la evaluación retrospectiva de i) el progreso en términos de rendimiento y ii) la magnitud y el efecto de la interacción genotipo-por ambiente del rendimiento en los materiales avanzados. La base de datos utilizada consiste en datos de rendimiento absoluto y relativo y fechas de floración de 349 líneas F8, F9 y F10, además de testigos, evaluadas en 163 ensayos distribuidos en 11 años. Las localidades de ensayo están en las provincias de Albacete, Lleida, Valladolid y Zaragoza. El progreso del programa se estimó utilizando el rendimiento relativo analizado mediante modelos mixtos para homogeneizar los resultados entre años y localidades, que son muy desequilibrados. Se constató la existencia de progreso en el programa, aumentando los rendimientos en cada generación, hasta superar a los testigos, aunque el progreso varió entre provincias y entre localidades. La ganancia genética fue mayor de F8 a F9 que de F9 a F10. El componente de varianza más grande (además de los puramente ambientales) fue el de genotipo por localidad y por año, por lo que los patrones geográficos eran imprevisibles. La relación entre fecha de floración y rendimiento en general fue débil en todas las localidades en esta etapa avanzada del programa. El programa puede continuar con la misma estructura, pero se debería investigar la causa del menor progreso obtenido en algunas localidades.

Palabras clave adicionales: *Hordeum vulgare*; interacción genotipo por ambiente; selección genealógica.

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This work has 1 Supplementary Table and 1 Supplementary Figure that do not appear in the printed article but that accompany the paper online.

Introduction

Barley, *Hordeum vulgare* L., is one of the most important cereal crops in the world (Baik & Ullrich, 2008), and it is grown in regions with climates unfavorable for production of other major cereals. It is commonly grown under dry conditions, poor and even saline soils, where it has a productive advantage. Because of these characteristics, it has been the principal grain produced in numerous stress-prone areas (Poehlman, 1985; Guttier *et al*., 2001), including the Mediterranean basin. In 2009, the barley cultivation area in Spain was 3.05 million hectares, and the production was 7.35 million tons, which corresponded to 22% of the total area devoted to barley in the European Union, and 13.5% of the total production (FAOSTAT, 2011). It is the first crop in terms of acreage in Spain, being mostly grown in dry inland areas.

Despite being such an important crop for Spain, the breeding activities carried out by private companies are almost non-existent. The reason is the low profit obtained from sales of seed, as less than 10% of the surface is sown to certified seed. As a consequence, most cultivars available to growers in Spain have been bred in other countries. Even though some of these cultivars perform quite well in Spain, we expect that local breeding should result in superior cultivars. Studies carried out in the Mediterranean region have demonstrated that the most effective way to improve productivity of crops grown in less-favored areas is to use locally adapted germplasm and select in the target environment(s) (Ceccarelli, 1994; Ceccarelli *et al*., 1998). The Spanish program takes advantage of this approach by local testing and also by the use of local landraces (Lasa, 2008) as source of adaptation traits.

Therefore, there was a need to provide Spanish growers with cultivars adapted to their local conditions. The Spanish National Barley Breeding Program was set out by four public research organizations with this purpose. These four centres are placed at the most representative barley growing regions of Spain. The program is conducted in a joint manner by four public research bodies: Instituto Técnico Agronómico Provincial (ITAP) in Albacete, Instituto de Investigación y Tecnología Agroalimentarias (IRTA) in Lleida, Instituto Tecnológico Agrario de Castilla y León (ITACyL) in Valladolid and Estación Experimental de Aula Dei (EEAD-CSIC) in Zaragoza (Fig. 1).

Figure 1. Location of the testing sites of the Spanish National Barley Breeding Program. Provinces (in grey) and locations (in black) hosting field trials.

The main objectives of this study were to study the progress and the selection efficiency in the Spanish National Barley Breeding Program, and to verify if this progress occurred uniformly across the four provinces of the program. Also, we wanted to have a general assessment of the extent and impact of genotype-by-environment interaction (GE) of grain yield in the final stages of the program. This study will focus on grain yield, the main target of the breeding program, but also on its relationship with flowering date. Flowering date is one of the most important traits for improving crop productivity and adaptation (Lawn *et al*., 1995; Laurie, 2009; Brachi *et al*., 2010), and is a primary objective of all breeding programs around the world.

Material and methods

Program description

The breeding program follows a strict pedigree scheme. Lines are extracted from the F2, and advanced up to the F10 following a head-row system. Early gen-

Abbreviations used: G (genetic gain); GE (genotype-by-environment interaction); GL (genotype-by-location interaction); GLY (genotype-by-year-by-location interaction); GY (genotype-by-year interaction); H (the realized heritability); REML (restricted maximum likelihood); S (selection differential).

eration testing takes place from F3 up to F5, independently at each site. F6 is the first generation of joint testing where the lines from the four provinces are merged together for testing. The advanced trials start in F7 and continue up to F10. The number of lines selected is reduced at each generation.

At each province, several locations were used for testing (Fig. 1). In Albacete two trials were carried out in the same location: Albacete dry-land (A1) and Albacete irrigated (A2). In Lleida, four locations were used: Artesa (L1), Bell-lloc (L2), Gimenells (L3) and Solsona (L4). In Valladolid, several locations were used: Castronuevo (V1), Geria (V1), Villabañez (V1), Zamadueñas (V1), Villahoz (V2), Ceinos (V3), La Espina (V4) and Macotera (V5). At Valladolid, four locations near the capital city were used in different years. These locations were close enough to each other to be considered as a single location, V1. And in Zaragoza two locations were used: Sádaba (Z1) and Vedado (Z2). For two years, a location from a neighboring province, Navarra, was used. This was coded as Z3, since it was close to the locations from Zaragoza (Fig. 1). Not all locations were used every year. Trials were rotated between locations, with the exception of Albacete, and usually there were two trials grown per province and year.

All the locations under study are non-irrigated locations, except Gimenells (L3), where irrigation was provided as needed to avoid losing the trial when drought was severe, and Albacete irrigated (A2), which was always under irrigation.

The temperature in the locations under study show patterns typical of the Mediterranean climate, but with some differences from location to location. Long term averages for temperature values were collected from the nearest meteorological stations to the locations under study.

Data set

The data of this study were collected from the advanced stages of the Spanish National Barley Breeding Program. The analysis focuses on the advanced generations of the program, with a low number of lines per generation (Table 1). In these advanced trials, grain yield was the main selection criterion. The data set was gathered from 163 trials corresponding to generations F8, F9 and F10 carried out from 1998 until 2008. A total of 349 advanced lines were studied during that period. Out of these, 327 were recombinant inbred lines derived from 197 hybridizations, and 22 were double haploid lines. Besides, up to 24 check varieties were evaluated in the trials (Table 1).

The trials of the advanced generations followed an alpha-lattice of variable block size, with three replications, embedded in a randomized complete block design, with several test lines and checks. Each plot occupied 7.2 m^2 (6 m × 1.2 m), with either 6 or 8 rows. This area was modified for this study to 10.5 m^2 (7 m × 1.5 m) to take into account border effects.

The traits considered were: raw grain yield (in kg ha^{-1}) at 10% moisture; relative grain yield for each line, expressed as the percentage of the average grain yield of the checks present at each particular trial; and flowering date, recorded as number of days from January

Table 1. Summary of lines and checks used in the advanced generations trials at the Spanish Barley Breeding Program

Years	Common checks	F8	F9	F10	F8	F9	F10
		Additional checks			Test lines		
1998	Barbarrosa, Alpha, Zaida	5	2	7	25	15	14
1999	Barbarrosa, Alpha, Zaida	2	2	6	20	11	7
2000	Barbarrosa, Alpha, Zaida, Graphic	1	6	6	23	4	4
2001	Barbarrosa, Alpha, Zaida, Graphic	2	6	6	30	6	1
2002	Barbarrosa, Alpha, Zaida, Graphic	1	1	2	23	15	6
2003	Barbarrosa, Alpha, Zaida, Graphic	0	1	2	32	15	12
2004	Barbarrosa, Alpha, Zaida, Graphic, Hispanic	0	0	0	31	15	11
2005	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	14
2006	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	11
2007	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	11
2008	Barbarrosa, Graphic, Hispanic, Cierzo	0	0	0	32	16	10
Total					312	145	101

1st when at least 2 cm of the awns were visible in 50% of the tillers of each plot.

The use of relative grain yield allows homogenizing the results among years and locations, and among analyses, therefore avoiding possible problems of scale due to differences in productivity across years and locations.

The data set is highly unbalanced because it was collected over 11 years, and the maximum period that any line stayed in the program was for three years. The advanced lines stayed in the program 1, 2 or 3 years, depending on the generation in which they were discarded. There were a few exceptions because some lines were introduced directly either in F9 or F10. For these lines, previous generations are missing. Also, a few lines were retained for additional years after F10, to get additional data before a final decision was made. To cope with the unbalancedness of the data, a mixed model approach (REML) was used, implemented in the software package Genstat 12 (Payne *et al*., 2009).

The relative grain yield was used to estimate the progress in the Spanish National Barley Breeding Program. To calculate the averages for each generation at each main location and province, two separate analyses were calculated using mixed models, considering locations or provinces as fixed factors, whereas years and the interactions with years were considered as random factors.

To calculate selection differential, genetic gain and realized heritability, the procedure of St. Martin & McBlain (1991) was used. The procedure is a test in which a set of lines evaluated in a generation is paired with a test in the next stage, in which selections from the set are re-evaluated. The procedure was adjusted to allow for the presence of different checks in the consecutive generations, which occurred in our data in some occasions. These calculations were done for the two selection steps available: F8-F9 and F9-F10, according to these expressions:

$$S = (X_S - X) \cdot 100$$
$$G = (X_S' - X) \cdot 100$$
$$H = G/S \cdot 100$$

where S is selection differential, X_s is the mean of the experimental lines selected from the first stage (F8 or F9) for testing in the successive second stage (F9 or F10), X is the mean of all experimental lines evaluated in the first stage (F8 or F9), G is genetic gain, X_S' is the mean of the experimental lines selected from the first stage and evaluated in second stage (F9 or F10) and H is the realized heritability.

To calculate the components of variance, the complete data set was used, but divided into two groups, according to the presence of a minimum of three common checks among the trials. The first group contained 242 genotypes and 12 locations during 7 years (1998-2004) and the second group contained 163 genotypes and 11 locations, during 4 years (2005-2008), with some genotypes represented in the two analyses. Even though the data were unbalanced, the presence of a minimum of common checks in all trials of each group of years, plus the presence of some breeding lines for two or three consecutive years, provided enough replication of genotypes to allow an estimation of variance components.

The components of variance were calculated using the original raw grain yield data. Genotypic averages per locations were used for these analyses, as these are the data available for all trials. For the sake of this analysis, genotypes, locations and years were considered as random factors, as they can be regarded as random samples of all possible levels of each factor that can be encountered for barley growing in Spain.

To break-down the GE into 'Genotype × Province' and 'Genotype within Provinces' interaction, two homogeneous series of genotypes repeated for two years were identified, *i.e.* 1998-1999, 2001-2002, 2003-2004, 2005-2006, and 2007-2008. Each series contained a group of genotypes tested in the same environments (combinations of years and locations) at two consecutive years. Analyses of variance (ANOVA) for relative grain yield were calculated for two series of balanced groups of genotypes. The first series contains the groups of lines in generations F8 and F9 at two consecutive years. And the second series contains groups of lines in generations F9 and F10 at two consecutive years. Each series contains five groups.

Linear regression was used to calculate the regression coefficient between flowering date and relative grain yield using the appropriate routine in Genstat 12.

Results

In all the advanced trials (F8, F9 and F10), several outstanding cultivars were included as checks. The number of checks varied from year to year, and also between locations, especially during the first years (Table 1). The checks were gradually changed along

the years, always aiming to include the best cultivars available, combining spring and winter cultivars. A set of common checks was maintained across locations, ranging from 3 to 5 checks per year. These common checks were chosen because they were used in the national trials for cultivar registration, and kept shifting as these cultivars were being renewed.

The selection pressure applied from generation to generation was not constant across years and, overall, was stronger at F8 (46% of lines promoted to F9) than at F9 (70% of lines promoted to F10).

The number of lines tested varied among years, with an average of 28, 13, and 9 lines tested in F8, F9, and F10, respectively (Table 1). In the period under study, a minimum of 31 genotypes were evaluated every year at advanced trials, at a minimum of three locations. Over the years, the program has become more stable in terms of number of checks and lines under test at every generation.

In the data set under study there was a large range in the grain yields recorded, from a minimum of 842 kg ha^{-1} to a maximum of 6,974 kg ha^{-1}. The overall mean for the entire period was 3,687 kg ha^{-1}. The productivity levels were quite different between locations. The least productive location was Albacete dryland (A1). The highest yielding location was Bell-lloc (L2). Productivity was also high in Gimenells (L3), Albacete irrigated (A2) and Macotera (V5), intermediate in Ceinos (V3), V1 (Castronuevo, Geria, Villabañez and Zamadueñas), Sádaba (Z1), Vedado (Z2) and Artesa (L1) (Table 2).

Across years, average productivity was less variable, always in the medium productivity range, from a minimum of 3,200 (2005) to a maximum of 4,890 kg ha^{-1}(2007). Productivity was higher in the last two years, in which it surpassed 4,000 kg ha^{-1}.

To estimate the progress due to selection, we needed to combine the results of years and locations, even though they had different productivity levels. For this purpose we used the relative yield, because it does not fluctuate across years and locations. Rather, it presents values always around 100, and so the values for all trials can be easily combined, although sacrificing the overall productivity perspective.

The averages, for each generation, at each main location and province were calculated in two separate analyses (one for locations, one for province, Table 2). Some of the locations were used only occasionally (L4, V2, V4 and Z3). Their inclusion in the analyses increased largely the unbalancedness of the data, therefore affecting the quality of any estimates derived from them. These minor locations were removed from most analyses to reduce the overall unbalancedness, and get better estimates of the factors studied for the main testing locations (Table 2).

Table 2. Grain yield expressed as percentage of checks and average productivity in different locations and provinces, in the last three generations (F8, F9 and F10) of the Spanish Barley Breeding Program from 1998 to 2008. Averages across provinces and overall average, calculated with REML, in bold type

	F8	F9	F10	Grain yield (kg ha^{-1})
A1	96.3	101.4	96.5	2,683
A2	98.1	101.4	105.9	4,517
Albacete	**96.0**	**100.7**	**100.8**	**3,626**
L1	101.2	101.9	102.5	3,012
L2	102.3	107.3	107.4	4,966
L3	99.4	94.5	98.4	4,636
Lleida	**101.1**	**101.3**	**102.8**	**4,179**
V1	99.0	100.8	97.4	3,478
V3	94.9	106.4	102.9	3,844
V5	98.6	101.6	105.3	3,900
Valladolid	**99.0**	**102.2**	**102.8**	**3,685**
Z1	97.2	101.6	105.4	3,138
Z2	101.8	110.7	113.5	3,021
Zaragoza	**97.6**	**103.3**	**107.5**	**3,109**
Total	**98.9**	**102.8**	**103.5**	

The comparison of the relative yields at the 10 main locations (during 11 years) indicated that there was progress at most locations over the three generations (Table 2). Overall, progress was evident. The means for the three advanced generations were different, F8 presenting the lowest mean and F10 the highest one (Table 2). At F8, the overall grain yield was already close to the level of the checks (98.9), and by F10 the outstanding lines clearly surpassed the checks by 3.5%.

Looking at the results of the provinces, in general, progress from F8 to F10 was observed at all four provinces, meaning that the program was successful overall. Differences among provinces were also apparent. The overall progress was larger at Zaragoza and Albacete, and smaller at Lleida and Valladolid.

Progress also differed at the single location level. In F8, only three of the ten main locations reached the yield level of the checks, whereas in F10 these figures were reversed. At F9, the progress was even more evident, as the lines surpassed the checks in all but one location. The highest progress was observed in Z2, where F10 lines surpassed the checks by 13.5%. The progress was large and consistent at the two Zaragoza

Table 3. Selection differential (S), genetic gain (G), and realized heritability (H, expressed as percentage of expected gain) calculated for groups of lines in two sets of consecutive generations (F8-F9 and F9-F10) tested in the same locations

	1st generation		2nd generation	S	G	H
	All lines	Selected lines				
F8-F9	95.9	102.1	102.0	6.24	6.09	97.6
F9-F10	99.9	106.1	100.2	6.28	0.37	5.9

locations, and smaller at the Lleida locations. In three locations, A1, V1, and L3 the average F10 lines did not reach 100, *i.e.,* their average did not surpass the checks'.

The selection differential (S), genetic gain (G), and realized heritability (H) were calculated for the two selection steps available: F8-F9 and F9-F10.The calculations of S, G and H, were done for sets of lines that were tested in the same location in consecutive years (Table 3). The figures indicate an excellent realized heritability was attained for the F8-F9 step, whereas it was low for the F9-F10 step.

The evaluation of a breeding program that includes testing in multi-environment trials must take into account which are the factors that cause genotypic variation. The relative size of these components will allow an assessment of the appropriateness of the testing strategies.

The components of variance were calculated for two subsets of data (Table 4), made of the sets of years that presented several common checks (Table 1). The component of variance for the error was calculated at each individual trial analysis, for each generation at each year and each location. These analyses are routinely done in the Spanish National Barley Breeding Program. The original data for all replicates was not always kept, but the original analyses of variance for most of them are still available. So, the error component of variance was calculated as an average of the error term corresponding to individual trials, weighted according to the degrees of freedom of each individual analysis.

After calculating the components of variance for the two groups independently, a weighted average was calculated for the components of these groups, relative to the number of units which were used in each analysis. This weighted average was assumed to represent the best estimate of the components of variance for the entire dataset under study.

The environmental components of variance were large. 'Location' was rather large, and 'Year' was highly variable. But, overall, 'Year × Location' was the dominant environmental component, which meant that the productivity of locations varied largely between years (Table 4).

The calculations of broad-sense heritability in the two analyses were 0.70 and 0.75 respectively, with a general average of 0.71 over the two analyses. These values suggest the possibility to perform selection effectively, though the response may be low some years due to a relatively low genotypic variance (Table 4).

An important variance due to 'Genotype' was present in the two analyses. The variance of the GL was larger than that of the GY in the two analyses. This suggests that GE shows some geographic trend. But the three way interaction (GLY) was larger or even much larger in each analysis, meaning that the geographic trends vary from year to year and are, therefore, unpredictable.

The GE was broken down into 'Genotype × Province' and 'Genotype within Provinces' interaction for the two balanced series of genotypes and environments. The analyses of variance for these groups are shown in Table 5. In most of the groups the variance of 'Genotype × Province' and the 'Genotype within Provinces'

Table 4. Components of variance for grain yield in the Spanish Barley Breeding Program. The two periods were chosen according to the presence of sets of common checks

Random term	1998-2004	2005- 2008	Weighted average
n (units)	2,172	1,865	
Year (Y)	0	1,657,120	765,551
Location (L)	1,073,410	1,158,223	1,112,592
Y × L	2,333,147	1,960,767	2,161,116
Genotype (G)	69,426	58,736	64,487
G × Y	95,698	26,570	63,762
G × L	145,824	34,329	94,316
G × L × Y	295,777	361,766	326,262
Error	208,858	235,394	224,711
Broad-sense h^2	0.70	0.75	0.71

Table 5. Summary of the genotype-by-environment interaction factor for ten different analyses of variance for relative yield. The analyses were performed for ten sets of genotypes, which were balanced over two-year trials, either F8 and F9 or F9 and F10

Years	Generations	Mean squares	
		Genotype × Province	Genotype within Province
1998-1999	F8-F9	253^{ns}	160
1998-1999	F9-F10	126^{ns}	234
2001-2002	F8-F9	91^{ns}	119
2001-2002	F9-F10	224^{ns}	141
2003-2004	F8-F9	182^{ns}	149
2003-2004	F9-F10	201^{ns}	190
2005-2006	F8-F9	95^{ns}	86
2005-2006	F9-F10	87^{ns}	111
2007-2008	F8-F9	102^{ns}	85
2007-2008	F9-F10	125*	69

terms were rather similar, and in 9 out of 10 of the groups the variance of 'Genotype × Province' (tested against the residual GE, *i.e.,* the 'Genotype within Provinces' term) was not significant. This means that, actually, the provinces did not explain much of the GE.

Flowering time data were recorded at most of the locations and years. When flowering date was recorded for a given location, it was done for all trials in that location. The averages of flowering dates for the three generations at all locations were calculated with a mixed model using REML, considering 'generation' and 'location' as fixed factors, and 'year' and its interactions as random factors (Table 6).

Lleida presented the earliest flowering dates, whereas the latest one was Valladolid. Zaragoza and Valladolid showed the widest flowering time ranges (Table 6). The flowering date means were almost constant across locations and provinces for the three generations F8, F9 and F10. The range of flowering dates became narrower with increasing generations, but this could be an effect of sample size.

The regression analysis between grain yield and flowering date was used to further analyze the possible presence of trends in the data. The regression coefficient was calculated using the relative yield and flowering time data of the genotypes under study (lines and checks). The regression coefficient was calculated for all trials run at each year-location combination (usually F8, F9 and F10, taking advantage of the fact that all three trials were commonly sown on the same date). The regression coefficients between relative grain yield and flowering time were low (Table 7). Even though it was statistically significant in some trials, due to the large number of points, the slope of the regression line was almost flat. In some trials (16, *i.e.* about one third), there was a significant negative relationship between relative grain yield and flowering time.

Table 6. Summary of number of lines, flowering date means, minimum, maximum, expressed as the number of days from January 1st, and range of flowering dates for the breeding lines under study (checks excluded), by location and province. Means are REML estimates, whereas minimum, maximum and ranges were calculated with raw values. Averages across provinces and overall average in bold type

	Lines	Mean	Minimum	Maximum	Range
A1	103	118.3	101	129	28
A2	101	121.7	105	140	35
Albacete	121	120.5	101	140	39
L1	119	114.1	96	127	31
L2	77	104.8	93	120	27
L3	99	106.3	89	119	30
Lleida	177	106.8	89	127	38
V1	93	126.2	110	142	32
V3	23	126.7	120	135	15
V5	121	120.0	108	135	27
Valladolid	135	123.3	108	142	34
Z1	159	120.4	108	141	33
Z2	69	114.1	96	130	34
Zaragoza	159	115.9	96	141	45
Total		117.3	102.6	131.8	29.2

Table 7. Results of the regression analyses between relative yield and flowering time in the trials during the period of the study

Location	Year	Generation	b	R^2	Constant	F pr.
A1	2003	F8-F10	–0.81	0.039	191	0.093
A1	2004	F8-F10	–0.30	0.009	135	0.427
A1	2005	F8-F10	–2.32	0.187	376	<0.001 **
A1	2006	F8-F10	–1.30	0.129	248	0.002 **
A1	2007	F8-F10	–2.44	0.106	412	0.006 **
A2	2003	F8-F10	–1.87	0.059	351	0.038 *
A2	2004	F9-F10	0.68	0.018	27	0.440
A2	2005	F8-F10	–0.12	0.002	112	0.748
A2	2006	F8-F10	–0.26	0.005	128	0.564
A2	2007	F8-F10	–3.32	0.127	523	0.002 **
A2	2008	F8-F10	0.34	0.006	56	0.536
L1	2003	F8-F10	–2.20	0.119	358	0.003 **
L1	2007	F8-F10	1.16	0.163	–25	< 0.001 **
L1	2008	F8-F10	0.89	0.075	–7	0.022
L2	1999	F8-F10	–1.63	0.187	270	< 0.001 ***
L2	2002	F8-F10	0.15	0.003	86	0.694
L2	2004	F8-F10	–1.46	0.052	272	0.053
L2	2006	F8-F10	–2.09	0.287	306	< 0.001 **
L3	1998	F8-F10	–1.00	0.030	209	0.135
L3	2000	F8-F10	0.33	0.010	57	0.517
L3	2001	F8-F10	0.16	0.002	87	0.746
L3	2005	F8-F10	0.13	0.001	80	0.781
L3	2007	F8-F10	0.08	0.002	89	0.709
V1	1998	F8-F9	–0.32	0.016	143	0.373
V1	2002	F8-F10	–0.10	0.001	111	0.788
V1	2005	F9-F10	–2.82	0.417	440	< 0.001 **
V1	2006	F8-F10	–0.69	0.016	195	0.312
V1	2007	F9-F10	–1.24	0.134	258	0.043 *
V1	2008	F8-F10	–0.26	0.013	129	0.345
V3	1999	F8-F10	–0.80	0.065	199	0.056
V4	1998	F8-F9	–0.77	0.060	219	0.079
V5	1999	F8-F10	–0.23	0.039	125	0.142
V5	2000	F8-F10	–1.56	0.365	296	< 0.001 **
V5	2002	F8-F10	0.14	0.004	82	0.619
V5	2005	F8-F10	–2.47	0.146	397	0.003 **
V5	2006	F8-F10	–3.30	0.321	485	< 0.001 **
V5	2007	F8-F10	–0.18	0.006	118	0.532
V5	2008	F9-F10	1.82	0.187	–110	< 0.001 **
Z1	2002	F8-F10	0.56	0.054	35	0.074
Z1	2003	F8-F10	–2.48	0.200	395	< 0.001 **
Z1	2004	F8-F10	–1.30	0.222	274	< 0.001 **
Z1	2005	F8-F10	–0.02	0.000	98	0.919
Z1	2006	F8-F10	–0.55	0.059	159	0.041 *
Z1	2007	F8-F10	–0.85	0.042	202	0.087
Z2	2001	F8-F10	–0.12	0.002	116	0.775
Z2	2003	F8-F10	0.25	0.001	82	0.750
Z2	2004	F8-F10	–0.45	0.006	174	0.539
Z2	2007	F8-F10	–0.46	0.037	155	0.107

*, **, significant at $p \leq 0.05$ and $p \leq 0.01$ respectively.

Discussion

The progress associated with selection, the relationship between flowering date and grain yield, and the existence of GE have not been studied previously in the Spanish National Barley Breeding Program. The success of the program is evident, based on its capacity to produce improved cultivars, which are being readily adopted by the industry and the producers. Nevertheless, a systematic retrospective analysis may offer clues about the effectiveness of the practices used, and help to identify possible weaknesses of the program.

It is assumed that each set of checks marked, at each year and location, the threshold of agronomic excellence for the program. Therefore, the overall relative yield means (Table 2) indicate a significant progress in the barley breeding program over the period studied. The difference between all three generations was remarkable, and in the end surpassed the yield of the checks. It seems that the overall progress slowed down after F9, however, as there was an increase of only 0.7% from F9 to F10 compared to 3.9% from F8 to F9. This may have been affected by the lower selection pressure applied from F9 to F10 (Table 1).

Another conclusion from the overall means is that the program already achieved a good productivity level at F8, with a mean performance quite close to the checks (98.9%). A similar trend in the performance of selected lines and check cultivars has been reported by Khalil *et al*. (2004) in a wheat breeding program. This may be the result of an efficient selection over the generations up to F8 or, alternatively, could mean that the productivity level achieved for the materials in the program is high from the very beginning. It is not inferred from the data which of these hypotheses is more likely. But the fact that most of the parents currently used in the program are recycled advanced lines suggests that the program may be reaching a mature stage, in which productivity level is optimized across all generations.

The true gain attained in the program is probably higher than the calculated for the relative yields. As the checks were gradually replaced over the years, it can be safely assumed that the yield level of the checks also rose over the years, as the new checks replaced older cultivars that became obsolete. In consequence, the gain calculated for relative yield is most likely an underestimation of the true gain in kilograms per hectare.

At the province level, there was higher progress in Albacete and Zaragoza, compared to Lleida and Valladolid. The small progress in Lleida and Valladolid may have been partially caused because, at these provinces, the F8 already showed a very high grain yield level, and subsequent progress could have been more difficult to attain. Though the gain in Albacete was apparent, the final yield level at F10 barely reached the level of the checks, whereas at the other three provinces, F10 lines level clearly exceeded the checks.

Gain from selection was apparent at most locations. In three locations, F10 relative yield was below 100, *i.e,* the program was less effective in finding superior cultivars for these locations. The case of V1 was not surprising, as it was actually a conglomerate of different locations close to Valladolid city and, in consequence, a larger effect of GE (lowering genetic gains) is expected. On the other hand, the case of A1 (Albacete dry-land) is worrying, as it seems that the program is not achieving its objective at the lowest yielding location. The low progress at this location affected the result of Albacete as a whole, and explains the unsatisfactory overall results at this province. It can be speculated that the program is not addressing properly the adaptation to the poorest growing conditions. To test this, we calculated a correlation coefficient between the program progress (the difference between F8 and F10) and the mean grain yield at the 10 main locations. The *r* value was just -0.12, indicating that the relationship between response to selection and productivity level was probably negligible. Finally, there is no plausible explanation for the low progress at L3.

Positive genetic gains from F8 to F9 were found (as in the studies of Khalil *et al*., 2004, 2010). But it was very low, almost negligible, from F9 to F10, though this was affected by other factor that will be discussed below. In any case, this indicates a lower effect of selection after F9. There were some lines tested for more than one year in F10. These lines used to be the best lines of the trial, that were maintained in the program for some additional years before taking the final decision of releasing them as cultivars or recycling them as parents. This was the reason of the apparently different results for the F10 in Table 2. In Table 3, the results of only the first year of F10 evaluation were presented. Actually, the lines that were kept in the program for additional years at the F10 had a relative yield above 105 in the second and third years of evaluation. Their absence in the calculations of realized heritability swayed the overall F10 average slightly downwards. The reasons for not reaching a realized heritability of 1 are the presence of error and of GE.

Regarding components of variance, 'Year' variance was very different between the two analyses done (Table 4). This is explained by the rather constant yearly averages observed during the first period analyzed (1998-2004), compared to the highly variable averages observed in the second period (2005-2008, Table 4). This was not unexpected, as large yearly fluctuations are common in Mediterranean environments (Turner, 2004). Genotypic variance was detected in the two analyses performed, meaning that there were true genotypic differences still at this stage of the program. It had comparable size to the GL and GY interactions. In a similar study focused on a wheat breeding program, Roozeboom *et al*. (2008) found a genotypic variance almost twice as large as the GL and GY variances. Similar figures were found by Thomason & Phillips (2006), for wheat breeding in Virginia. Their studies are relevant to ours because they were also testing advanced materials (candidate cultivars) in large geographical areas with highly variable environments (especially Roozeboom *et al*., 2008). This shows that the situation for the Spanish barley breeding program presents even higher challenges, as the interactions involving the 'Genotype' factor were higher.

GL in the data was rather high, indicating the presence of a geographical factor in the GE. When this happens, the breeders are confronted with the issue of whether the program should target wide adaptation, or it should be split between different locations due to the high GL interaction. But the results in the two analyses comprising the entire 11 years (Table 4) indicate that the 3-way interaction, between genotypes, locations and years was the principal source of variance. Therefore, the geographical patterns varied between years and were not predictable. Hence, a split of the program based on more stable geographic sub-zones is not advisable.

Consistent with this, it is observed that there was almost no Genotype × Province interaction (Table 5). Therefore, whatever factors were causing GE in this dataset, they seemed not related with geographical division at the province level. This finding reassures that the current strategy, combining the results of the four provinces is appropriate. Cullis *et al*. (2000) found a similar situation when analyzing series of variety tests conducted for several crops in Australia. They found that classical geographic zonation had little meaning under the light of actual variance components calculated for them.

The presence of locations from all provinces ensures a good coverage of all GE situations possible. In other words, the representativeness of the locations is good. It may be argued that the two Albacete locations (actually, two trials in the same location) are redundant to some extent. But the very distinct results observed in response to selection between A1 and A2 (Table 2) suggests that these two trials are probably giving different, non-overlapping information.

The changes in flowering date means and ranges indicate that, even though this trait has undergone several rounds of selection by this stage of the breeding program, there was still a slight selection towards earliness from F8 to F10 (Table 6). There was a spread of flowering dates across locations, proportional to the mean temperatures over the growing season, with colder locations (from Valladolid) reaching flowering later than warmer locations (for instance, L2 and L3). A dynamic relationship of flowering date with barley yield in Spanish environments was already found by Cuesta-Marcos *et al*. (2009). Though some water stress is almost always present in our conditions, timing and intensity of this stress varies widely. Therefore, it is not surprising that the relationship between flowering date and yield changed depending on the environment. The regression coefficients between relative grain yield and flowering time were, in general, rather low (Table 7) indicating that the relationship between yield and flowering time overall was weak in the locations under study at this advanced stage of the program. This relationship would possibly be more tight if the selection up to F8 had not removed already the most early and, especially, late genotypes.

In summary, there was progress due to selection over the last generations of the Spanish National Barley Breeding Program. Grain yield increased from F8 to F10, surpassing the level of the checks. We can conclude that the program is reaching its main goal of producing and identifying superior barley genotypes with high yield potential and stability suitable across all Spanish barley growing regions. The effectiveness of selection was satisfactory across all four provinces, though differences were observed among particular locations. It was also more effective up to F9, whereas there was little gain in the last generation.

These results also suggest that it would be unpractical to run separate breeding programs for separate provinces or locations (either considering an entire program or just the last generations). If we had found clear differences in GE among provinces, the situation might have been different, as provinces are large geographical units, which may justify additional efforts. But the struc-

ture of the components of variance and the absence of a stable geographic structure of the GE, it seems sensible that the program continues with the same geographic structure, using the same provinces and locations.

The definitive proof of the success of a breeding program is the adoption of the varieties released by the industry. Cultivars Cierzo, Estrella and Yuriko, released over the last five years performed very well in independent trials, and are currently under exploitation by three different companies.

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