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Association of SNP Markers with Agronomic and Quality Traits of Field Pea in Italy

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Abstract

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Only a few studies on pea (*Pisum sativum*) investigated the association of single nucleotide polymorphisms (SNP) markers with key agronomic traits. This study aimed to explore the association of a standard set of 384 SNP with grain yield, seed protein content, seed weight, onset of flowering, plant height and lodging susceptibility, in three connected bi-parental recombinant inbred line (RIL) populations including 90 lines each. These RIL originated from crosses between three cultivars that displayed high and stable grain yield across Italian environments, namely, Attika (A), Isard (I), and Kaspa (K). The 270 lines were phenotyped in a spring-sown environment of Lodi (northern Italy; 45°19'N, 9°30'E). Variation among lines within the populations was significant ($P < 0.01$) in all cases except lodging susceptibility in one cross and, when expressed in terms of the genetic coefficient of variation, proved moderately large for most traits (including grain yield and seed protein content). Overall, we detected six quantitative trait loci (QTL) in the A × I linkage map, eight QTL in K × A, and nine QTL in K × I. Among them, there were three QTL in K × A and two QTL in K × I for grain yield, and one QTL in A × I and two QTL in both K × A and K × I for seed protein content. The consensus map, which included 130 markers (covering about 1094 cM), retained one QTL for grain yield and one for flowering time that co-located on LGII, and three for seed weight on LGIII, LGVI and LGVII. The QTL co-locating for yield and flowering time explained 8% and 31% of the overall phenotypic variation, respectively, for the two traits, and could be exploited in marker-assisted selection for adaptation to the target region.

Keywords: grain yield; marker-assisted selection; *Pisum sativum*; protein content; QTL

Grain legumes can greatly contribute to more sustainable agriculture in Europe with regard to soil fertility, diversity of cultivated plants, nitrogen balance, energy consumption and greenhouse gas emissions, while providing feed and food rich in protein, energy and bioactive compounds (Carrouée *et al.* 2003; Nemecek *et al.* 2008). However, the cultivation of legume crops in Europe is declining (FAO 2013). The profitability of these crops in European farming systems may emerge when considering all relevant factors within a rotation (Von Richthofen *et al.* 2006), but depends crucially on the ability to increase the current crop yields (Schreuder & De Visser 2014).

Field pea (*Pisum sativum* L.) has a particular interest as a feed crop in southern Europe, owing to its ability to attain higher yield levels relative to other grain legumes that emerged in comparisons of spe-

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cies represented by locally top-yielding cultivars (Annicchiarico 2008). High grain yield and yield stability via optimal phenology (to escape prevailing climatic stresses), tolerance to lodging and major biotic stresses, as well as high seed protein content, are key traits for pea improvement as a feed crop (Huyghe 1998; Duc *et al.* 2015). Marker-assisted selection (MAS) may contribute to improve these complex, polygenic traits and reduce the need for costly field selection trials. However, the identification of quantitative trait loci (QTL) and linked markers exploitable for selection is hindered by the fact that the pea genome is very large (Ellis & Poyser 2002) and not yet sequenced. Several pea molecular linkage maps have been constructed by integrating different types of markers, such as RFLP, AFLP, STS, SSR, RAPD and/or CAPS (Weeden *et al.* 1998; Irzykowska *et al.* 2001; Tar'an *et al.* 2003, 2004; Timmerman-Vaughan *et al.* 2005). Krajevski *et al.* (2012) used AFLP, RAPD, STS, CAPS and ISSR markers to localize QTL for yield components and protein content of two sets of recombinant inbred lines (RIL), reporting loci with consistent or inconsistent effects. Single nucleotide polymorphisms (SNP) have now become the preferred markers due to their abundance and uniform distribution throughout genomes (Gupta *et al.* 2008), as confirmed by molecular linkage maps produced by Aubert *et al.* (2006), Deulvot *et al.* (2010), Duarte *et al.* (2014) and Sindhu *et al.* (2014). However, investigations on the linkage of SNP markers with pea production, phenology or grain quality traits are relatively few (Timmerman-Vaughan *et al.* 2005; Burstin *et al.* 2007; Klein *et al.* 2014; Cheng *et al.* 2015; Jha *et al.* 2015), and the QTL ability to explain sufficient phenotypic variation for use in MAS is controversial. One reason for that is the complex and polygenic control of most of these traits, which results in only moderate cumulative QTL effect (Tar'an *et al.* 2004; Burstin *et al.* 2007; Klein *et al.* 2014).

The objective of this study was to explore the ability of a standard set of SNP markers developed at INRA (Deulvot *et al.* 2010; Carrillo *et al.* 2014) to identify QTL for major agronomic and qualitative traits of pea in three connected bi-parental populations that were phenotyped in a spring-sown cropping environment of northern Italy. The three parent lines were elite varieties selected from a large number of recent cultivars on the grounds of high and stable grain yield across several Italian test environments (Annicchiarico 2005; Annicchiarico & Iannucci 2008). Bi-parental populations originated from top-performing parent lines are likely to be more challenging for QTL identification than those issued from parents with contrasting yielding ability, but they reflect far more closely the context in which MAS can be of interest for actual breeding programmes.

MATERIAL AND METHODS

Plant material. Three connected RIL populations originated from paired crosses between Attika (an European cultivar described as a spring-type), Isard (a French winter-type cultivar) and Kaspa (an Australian cultivar, phenologically intermediate). These cultivars, while being geographically diverse, displayed fairly similar phenology and cycle duration along with good yielding ability and other agronomic characteristics (e.g., tolerance to lodging) across environments of northern and southern Italy (Annicchiarico 2005; Annicchiarico & Iannucci 2008). The three RIL populations, termed hereafter as A × I, K × A and K × I according to the initials of their respective parents, were developed from F_2 seed by single-seed descent, advancing 90 RIL per cross. Four F_6 plants per line were grown in an unheated glasshouse, to collect DNA samples for line genotyping and to produce the seed used for phenotypic evaluations.

Phenotypic data. The 270 RIL issued by the three crosses were evaluated in a field trial carried out in Lodi (45°19'N, 9°30'E), northern Italy, a site with subcontinental climate and sandy loam soil. The experiment was sown on April 2, 2012 according to an alpha lattice design with two replications. Spring sowing is traditionally preferred in northern Italy to avoid the possible occurrence of severe mortality due to low winter temperatures (Annicchiarico & Iannucci 2007). Each plot included 40 seeds sown in 4 rows 1-m long and 25 cm apart. Pre-sowing fertilization provided 32 kg/ha of N and 96 kg/ha of both P_2O_5 and K_2O. Pre-emergence chemical weed control was carried out by applying 2.5 l/ha of pendimethalin. A treatment with lambda-cyhalothrin (100 ml of commercial product in 100 l of water) was applied during seed formation to prevent bruchid (*Bruchus pisorum* L.) seed infestation. It was a rainfed trial that received 218 mm of rainfall over the crop cycle.

The following traits were recorded on a plot basis: (*i*) grain yield, expressed at 7% humidity after seed moisture determination; (*ii*) individual seed weight;

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(*iii*) seed protein content; (*iv*) onset of flowering, as number of days from May 1; (*v*) plant height at the onset of flowering; and (*vi*) lodging susceptibility at physiological maturity, measured on a 1–5 visual scale where 1 = upright and 5 = completely lodged. Protein content was determined on samples ground at 0.2 mm with a ZM100 Retsch grinder (Retsch GmbH, Haan, Germany), using a NIRS 6500 equipment with autosampler module (Foss Tecator, Höganäs, Sweden). The calibration curve was developed by INRA of Dijon and conveniently optimized for our samples in this project. Validation results for a subset of 23 samples analysed by the Kjeldahl method (NF EN ISO 20483, January 2007) showed a very high predicting ability of the curve, with a slope of 1.022 and R^2 = 0.985 (SE of prediction = 0.324, and bias = 0.093).

Phenotypic data were subjected to analysis of variance (ANOVA) to assess variation between lines of each RIL population. A second ANOVA compared RIL populations, holding the pooled variation among lines within population as the error term. Genotypic and experimental error variance components were estimated for each RIL population, to estimate broad-sense heritability on an entry mean basis (h^2) and the genetic coefficient of variation (CV_g, as the ratio of genotypic standard deviation to mean value) for each trait. Finally, we computed phenotypic correlations between traits within each RIL population. All of these statistical analyses were carried out using Statistical Analysis Software (SAS Institute Inc., Cary, USA).

DNA extraction. Green tissue for DNA extraction was collected from stipules of the parent lines and from bulked stipules of the four F_6 plants per line grown in a glasshouse. The plant material was flash frozen in liquid nitrogen, stored at –80°C and then ground for the subsequent total genomic DNA isolation.

DNA was extracted from 400 mg of tissue using a CTAB method as described by Rogers and Bendich (1985), and then checked on 1% agarose gel and by means of a JASCO V-530 spectrophotometer (OD260/OD280) (JASCO, Inc., Tokyo, Japan) to assess DNA yield and quality.

Genotyping data. GoldenGate® genotyping assay with VeraCode® Technology on a BeadXpress platform (Illumina, San Diego, USA; see Gupta *et al.* (2008) and Deulvot *et al.* (2010) for a detailed description) was organized in a 384 SNP array which allowed to screen all markers in parallel. Imaging and data analysis were performed by GenomeStudio package (Illumina Inc., San Diego, USA). The discovery of SNP into genes was previously carried out by searching in public databases for plant gene sequences (encoding mainly proteins involved in development, carbon/nitrogen/amino acid metabolisms, transcriptional regulation and transport), designing primers around or close to the polymorphic sites in segregation analyses of parents. Some of them were selected among those listed by Bordat *et al.* (2011), whereas others were added to ensure optimal genome coverage. All SNP names, details and sequences can be found in Additional file 2 by Deulvot *et al.* (2010) and in Supplementary material 2 by Carrillo *et al.* (2014). Homozygous or heterozygous genotypes for each marker were displayed in different clusters, editing cluster analysis results when needed using Caméor (a European garden pea variety) as internal genotyping reference. Markers gave consistent genotyping results when more than one SNP per gene was assayed, confirming the accuracy of the method and the absence of recombination events in the gene sequence. In such cases, we kept only one relevant SNP for the map construction.

The MAPMAKER/EXP version 3.0b computer package (Lander *et al.* 1987) was used to build genetic maps and to localize the loci along the seven chromosomes. Linkage maps were generated for each RIL by MapChart 2.2 software (Voorrips 2002), after discarding markers that exhibited segregation distortion. A consensus map was built, considering non-segregating markers (i.e., the monomorphic ones in a given population) as missing data. Calculations of centiMorgan (cM) distances between each pair of markers were performed by the Kosambi function converting recombination frequency values. QTL analyses between phenotyping and SNP were performed by the MCQTL software (Jourion *et al.* 2005).

RESULTS AND DISCUSSION

Phenotypic data. Variation between crosses in mean values of the RIL populations was detected for all traits except individual seed weight and seed protein content (Table 1). On average, the lines issued by the A × I cross exhibited higher grain yield and earlier onset of flowering, whereas those issued by K × A combined intermediate yield, taller plant, and lower susceptibility to lodging (Table 1). Variation among lines within population was statistically significant ($P < 0.01$) in all cases except lodging susceptibility in K × A material and, when expressed in

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Table 1. Mean value, broad-sense heritability on an entry mean basis (h^2) and genetic coefficient of variation (CV_g) for phenotypic traits of three connected pea RIL populations (A × I; K × A; K × I)

Trait	Mean			h^2			CV_g (%)		
	A × I	K × A	K × I	A × I	K × A	K × I	A × I	K × A	K × I
Grain yield (t/ha)	3.50[a]	2.51[b]	1.68[c]	0.73	0.90	0.62	31**	61**	47**
Seed protein content (%)	22.0[a]	22.4[a]	22.0[a]	0.84	0.83	0.88	5**	6**	8**
Individual seed weight (g)	0.163[a]	0.163[a]	0.156[a]	0.77	0.87	0.69	12**	15**	11**
Onset of flowering (days from May 1)	26.0[a]	29.0[b]	28.0[b]	0.83	0.94	0.75	7**	13**	12**
Plant height (cm)	66.4[a]	72.1[b]	65.4[a]	0.85	0.73	0.76	13**	11**	14**
Lodging susceptibility (1–5)	2.6[a]	1.6[b]	2.2[c]	0.61	0.22	0.66	48**	20[ns]	49**

Population means followed by different letters differ at $P < 0.05$ according to Newman-Keuls test; [ns], **genotypic component of variance not different from zero ($P > 0.05$) and different from zero at $P < 0.01$, respectively

terms of the genetic coefficient of variation, it proved large for grain yield (CV_g > 30%) and moderately large for most other traits (Table 1). Broad-sense heritability values indicated that genetic variation, when significant, was the main determinant of the recorded phenotypic variation among lines within each population ($h^2 \geq 0.61$ in almost all cases; Table 1). The RIL issued by K × A were characterized by outstanding genetic variation (CV_g = 61%) and broad-sense heritability (h^2 = 0.90) for grain yield (Table 1). Comparatively higher values of both CV_g and h^2 were displayed by K × A lines also for seed weight and onset of flowering, and by K × I lines for tolerance to lodging and seed protein content (Table 1). Although not very large in terms of absolute CV_g values, RIL variation for protein content was

Table 2. Correlations between phenotypic traits for three connected pea RIL populations (A × I; K × A; K × I)

Trait	Grain yield	Seed protein content	Individual seed weight	Onset of flowering	Plant height
A × I					
Seed protein content	0.07[ns]	–			
Individual seed weight	0.42**	–0.16*	–		
Onset of flowering	–0.29**	0.16*	–0.12[ns]	–	
Plant height	0.28**	–0.03[ns]	0.07[ns]	–0.24**	–
Lodging susceptibility	0.24**	0.48**	–0.04[ns]	0.07[ns]	–0.13[ns]
K × A					
Seed protein content	0.06[ns]	–			
Individual seed weight	0.52**	–0.05[ns]	–		
Onset of flowering	–0.78**	0.19**	–0.43**	–	
Plant height	0.29**	0.28**	0.27**	–0.13[ns]	–
Lodging susceptibility	0.19*	0.20**	0.08[ns]	–0.11[ns]	0.01[ns]
K × I					
Seed protein content	0.31**	–			
Individual seed weight	0.14*	–0.19*	–		
Onset of flowering	–0.49**	0.09[ns]	–0.32**	–	
Plant height	0.56**	0.41**	0.01[ns]	–0.26**	–
Lodging susceptibility	0.22**	0.30**	–0.01[ns]	–0.08[ns]	0.04[ns]

[ns], *, **not different from zero and different at $P < 0.05$ and $P < 0.01$, respectively

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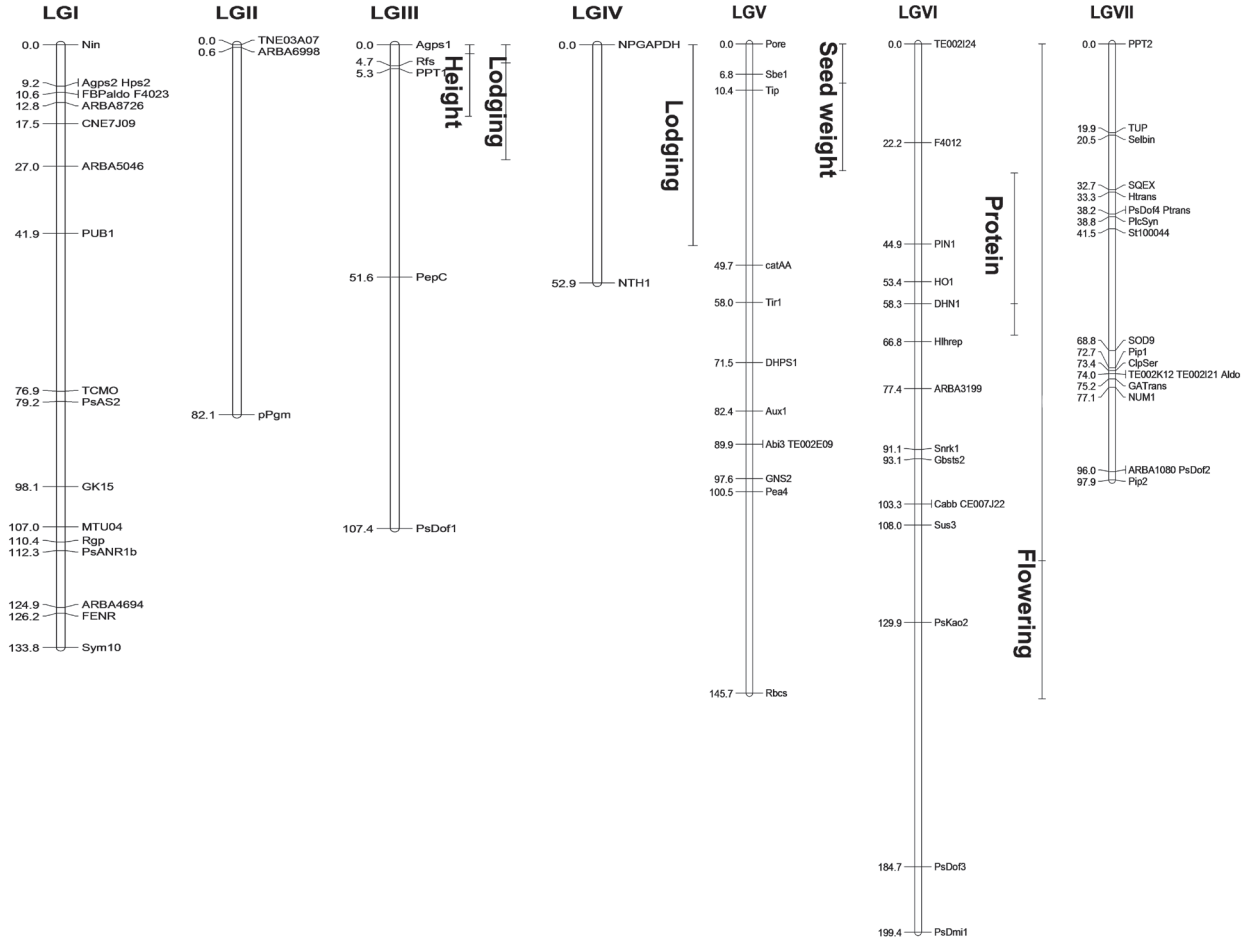

Figure 1. Linkage map for Attika × Isard population, and QTL detection

moderately large. Its overall range of variation, i.e. 17–27%, was comparable with that reported in Jha *et al.*'s (2015) study, and larger than in most other reports (e.g., Smýkal *et al.* 2012).

Correlation coefficients among phenotypic traits are reported for each population in Table 2. In all populations, higher grain yield was consistently associated with heavier seeds, earlier onset of flowering, taller plant at the onset of flowering, and higher lodging susceptibility (although this latter association was always loose). Relatively high correlations between the remaining traits were occasional and inconsistent across the three sets of RIL. The association of higher grain yield with earlier flowering, which was higher in the two populations including Kaspa as the parent, reflected the usefulness of early phenology as an escape strategy from terminal drought and high temperatures of the test environment.

Higher grain yield was associated negatively to protein content only in the K × I population, while showing no correlation in the other crosses. This finding is promising for the simultaneous improvements of both traits in this material. A negative correlation between grain yield and protein content has often been reported in other studies, e.g., Tar'an *et al.* (2004) and Krajewski *et al.* (2012).

Genotypic data and QTL search. We identified seven linkage groups for each RIL population (LGI to LGVII) corresponding to the seven pea chromosomes, which are reported in Figures 1, 2 and 3. Polymorphic SNP markers were 75 (along 819 cM) for the A × I population, 81 (along 814 cM) for K × A, and 95 (along 936 cM) for K × I. The consensus map included 130 markers (24 on LGI, 12 on LGII, 20 on LGIII, 12 on LGIV, 18 on LGV, 19 on LGVI, and 25 on LGVII; Figure 4), covering about 1094 cM overall.

On the whole, we detected six QTL in the A × I linkage map, eight QTL in K × A, and nine QTL in K × I. Five QTL were retained in the consensus map, namely, one for yield and one for flowering time on LGII, and three for seed weight on LGIII, LGVI and LGVII (Figure 4). For grain yield, we found three QTL

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in K × A (Figure 2) and two QTL in K × I (Figure 3). The QTL retained in LGII of the consensus map, which exhibited a negative value in Kaspa, explained a remarkable amount of variation for this quantitative trait in K × A (R^2 = 0.45) but the modest variation in K × I (R^2 = 0.11; Table 3) and, overall, limited variation in the consensus map (R^2 = 0.08; Table 4). LGII harboured one QTL for grain yield also in Tar'an *et al.* (2004) and Krajewski *et al.* (2012).

We detected some QTL for seed protein content in the individual populations, i.e., one on LGVI in A × I, and two on LGIV and LGVII in both K × A and K × I (Figures 1–3). The association of the *Htrans* marker (encoding a transporter for hexose) with the QTL in LGVII emerged also in the study by Burstin *et al.* (2007). However, no QTL for protein content was retained in our consensus map, probably because of insufficient robustness deriving from large confidence intervals (Figures 1–3).

For the onset of flowering, we found one QTL in LGVI for A × I, one QTL in LGII for K × A, and three QTL in LGII, LGIV and LGVI for K × I (Figures 1–3). The QTL in LGII that was retained in the consensus map explained a relatively high amount of variation for this trait in the relevant populations K × A and K × I (R^2 > 0.48; Table 3) and the consensus map (R^2 = 0.31; Table 4), and expressed a later-flowering QTL provided by Kaspa (Table 3). This QTL co-located with the QTL for grain yield in the consensus map (Figure 4), reflecting the high inverse correlation of flowering date with grain yield that featured the two populations including Kaspa as a parent line (Table 2). Two markers were associated with these QTL, namely, Cwi1 for cell wall invertase 1 and ARBA1788 for arabinase A. One QTL for flowering time was reported in about the same position of LGII by Fondevilla *et al.* (2008), whereas a few QTL for developmental traits were reported on LGII

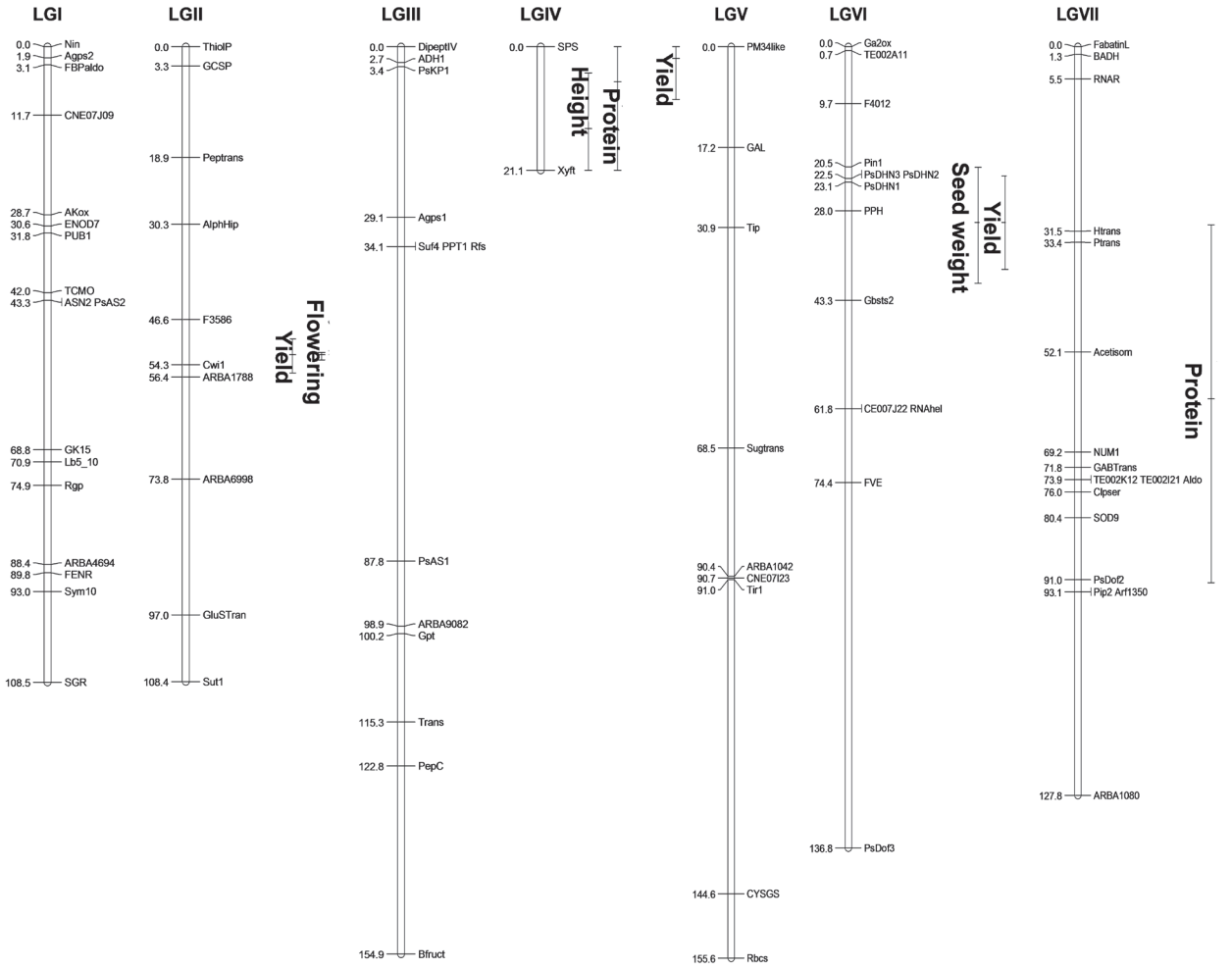

Figure 2. Linkage map for Kaspa × Attika population, and QTL detection

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Table 3. Position in linkage group II and value of QTL for yield and flowering time of two pea RIL populations

Trait	Population	QTL position (cM)[a]	Confidence interval (cM)[b]		*P*-value[c]	R^{2d}	Allelic value	Parental assignment
Yield	K × I	91.5	0.0	100.6	2.71	0.11	–0.14	Kaspa
	K × A	52.6	49.9	55.7	11.26	0.45	–0.45	Kaspa
Flowering time	K × I	46.1	42.6	53.7	13.05	0.49	1.45	Kaspa
	K × A	52.6	52.2	53.5	27.11	0.77	1.89	Kaspa

[a]QTL position from the first marker of the linkage group (in cM Kosambi); [b]position of the lower and upper ends of the QTL confidence intervals, from the first marker of the linkage group (in cM Kosambi); [c]peak *P*-value at the QTL position for each variable; [d]phenotypic variance explained by each QTL

by Timmerman-Vaughan *et al.* (2005). The high variation for flowering time explained by the QTL on LGII is consistent with the report of a major late-flowering gene on this linkage group (Arumingtyas & Murfet 1994). The genetic control of flowering was also studied by Weller *et al.* (2009), who identified loci and candidate genes in garden pea using mutant isolation and expression analyses.

We detected three QTL for individual seed weight on LGIII, LGVI and LGVII of the consensus map (Figure 4). The position of the QTL on LGIII (Table 4) is roughly coincident with that reported by Burstin *et al.* (2007). Despite their number, the combined ability of these QTL to explain the trait variation was moderately low (R^2 = 0.16; Table 4). The QTL for this trait was observed in LGVI of the

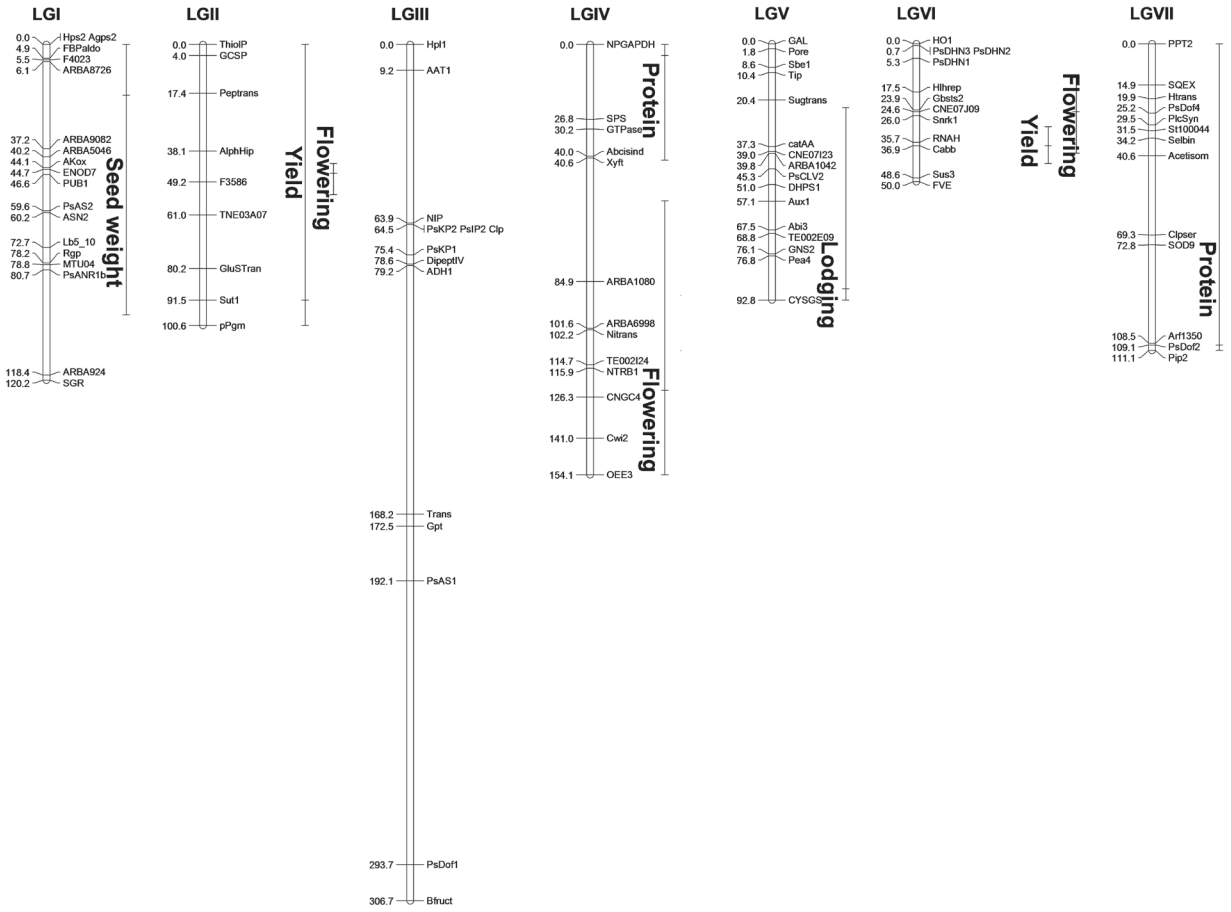

Figure 3. Linkage map for Kaspa × Isard population, and QTL detection

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Table 4. QTL parameters for three traits in the consensus map for the three connected pea RIL populations

Trait	Linkage group	QTL position (cM)[a]	Confidence interval (cM)[b]		*P*-value[c]	R^{2d}
Grain yield	LGII	54.3	38.0	62.7	5.5	0.08
Onset of flowering	LGII	54.3	50.5	72.4	21.6	0.31
Individual seed weight	LGIII	158.6	157.7	168.6	3.6	0.05
Individual seed weight	LGVI	103.8	98.6	113.4	4.0	0.06
Individual seed weight	LGVII	118.1	21.7	162.1	3.6	0.05

[a]QTL position from the first marker of the linkage group (in cM Kosambi); [b]position of the lower and upper ends of the QTL confidence intervals, from the first marker of the linkage group (in cM Kosambi); [c]peak *P*-value at the QTL position for each variable; [d]phenotypic variance explained by each QTL

population K × A co-located with a QTL for grain yield (Figure 2), probably contributing to the positive correlation between these traits (which tended to be higher in this population: Table 2).

QTL for the remaining two traits were detected in population-specific linkage maps but not in the consensus map. In particular, we observed QTL for lodging susceptibility on LGIII and LGIV in A × I, and on LGV in K × I (Figures 1 and 3). The QTL on LGIII co-located with plant height (Figure 1), although in the absence of a correlation between these traits (Table 2). A pair of co-located QTL for lodging sus-

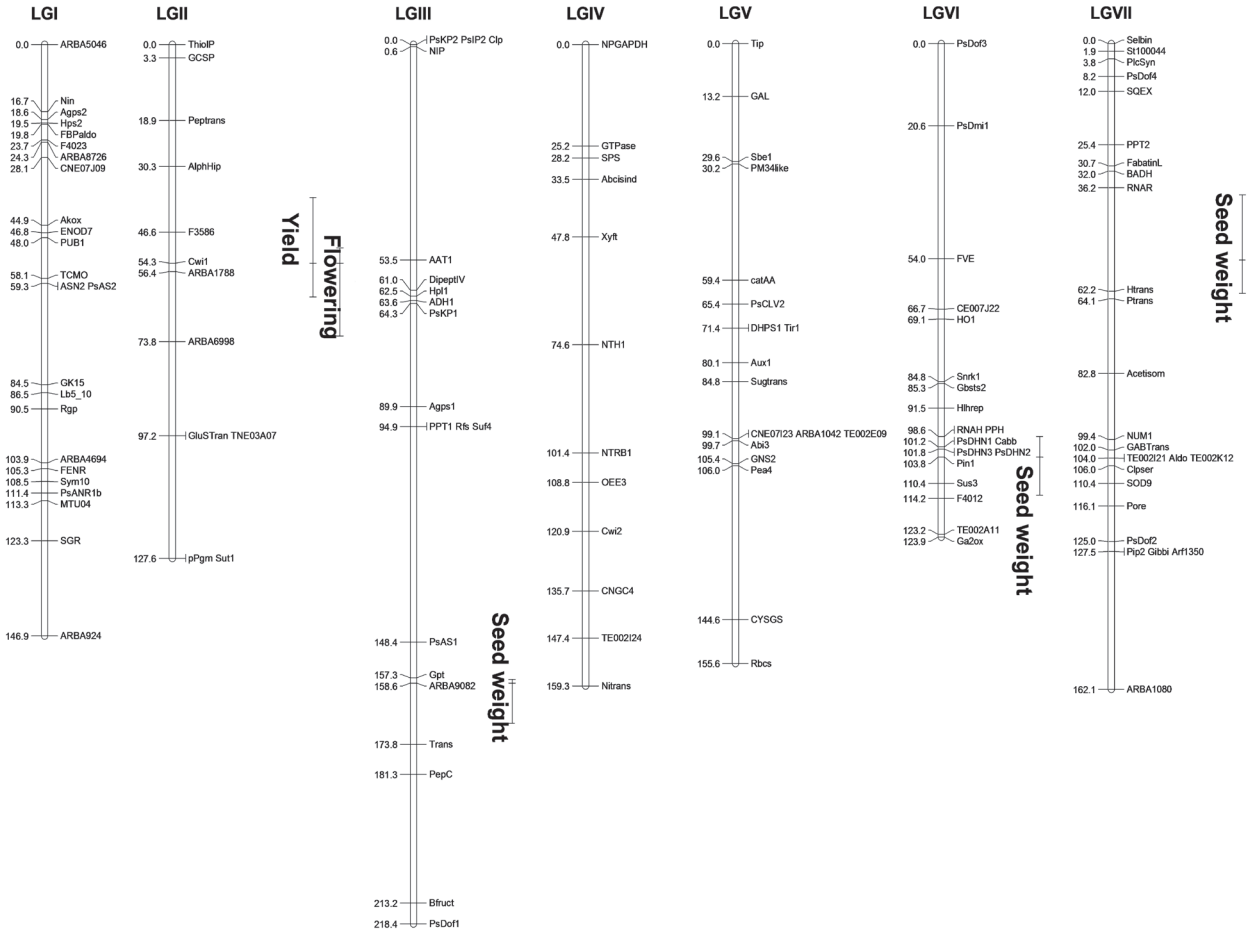

Figure 4. Consensus map for three connected pea RIL populations (A × I, K × A and K × I) with QTL detection

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ceptibility and plant height on LGIII was also found by Tar'an *et al.* (2003). We observed a second QTL for plant height on LGIV in K × A (Figure 2).

Our study highlighted a few QTL, such as those for flowering time and grain yield that co-located on LGII, which could be the object of marker-assisted selection, either via the current markers or through markers more closely associated to be identified through a fine-mapping approach. Our study suffered from low resolution in large chromosomal regions, owing to the limited number of anchored polymorphic SNP markers. This agrees with earlier studies (e.g., Timmerman-Vaughan *et al.* 2005), which, like this report, revealed useful genomic regions that could be explored through higher resolution mapping as allowed for by increasingly available genomic resources (Duarte *et al.* 2014; http://www.coolseasonfoodlegume.org/pea_genome). The inconsistent presence of QTL across mapping populations may arise, besides the effect of different genetic background, also from the insufficient marker accuracy with respect to QTL whose explanatory ability, even when detected, was usually limited. Low explanatory ability for key agronomic traits such as grain yield or protein content can be expected from their polygenic control. Contributing reasons for the relatively low number of QTL that we detected for various traits were the relatively low number of tested lines per individual RIL, and the anticipated adoption of agronomically outstanding parent cultivars (in agreement with the ordinary crossing strategy of breeding programmes). For example, the three parent cultivars (Attika, Isard, Kaspa) exhibited almost identical, high levels of mean grain yield and tolerance to lodging across a set of environments of northern Italy in earlier testing (Annicchiarico 2005). The similarity between parent lines did not prevent, however, the occurrence of large variation in grain yield among lines of each RIL population.

Detecting and taking account of many relevant small-effect QTL will only be possible by using high numbers of markers, as foreseen through the development of next-generation sequencing approaches for this crop based on SNP Array technology (Deulvot *et al.* 2010; Burstin *et al.* 2015). The adoption of GenoPea 13.2K SNP Array recently exhibited high genome-enabled predicting ability for traits such as thousand seed weight and flowering time (Tayeh *et al.* 2015), Genotyping-by-sequencing procedures (Elshire *et al.* 2011) may prove an alternative, possibly less expensive approach to the genomic prediction of quantitative traits, as indicated by preliminary results of grain yield prediction in different cropping environments for material that is largely coincident with that included in this study (Annicchiarico *et al.*, unpublished data).

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