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Development of a selection tool for seed shape and QTL analysis of seed shape with other morphological traits for selective breeding in chickpea (*Cicer arietinum* L.)

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Abstract

Chickpea (*Cicer arietinum* L.) is an annual diploid (2n= 2x= 16) grain legume, grown worldwide for human consumption. Selection for variation in the physical seed characters of chickpea will enable future strategic breeding of varieties with the potential to attract premium prices in overseas markets. Seed shape is therefore a major current breeding objective, for which an understanding of the genetics of inheritance is required. For this, two recombinant inbred line (RIL) populations derived from intraspecific crosses of a kabuli-type (S95362; light cream colour) crossed to two desi-types (Howzat and ICC3996; medium tan and dark tan colour, respectively) were studied. In order to discretely characterize seed shape, a new Roundness Index (RI) tool was developed, calculated from the ratio of two seed size indices (SSI). The genetic parameters estimated were genotypic and total phenotypic variance for RI, genotype vs environment interaction and broad sense heritability. The low genotype x environment interaction (<9% of total variation for both populations) and high magnitude of heritability suggested the environmental stability of the seed shape trait. Segregation ratios for different seed shape among the RIL populations indicated control of seed shape under two genes. Subsequently, one putative quantitative trait loci (QTL) was identified on linkage group LG 2 between markers TA110-TA27 and accounted for 9% of the phenotypic variance. One QTL for stem colour and flowering time also detected on LG 2 between markers TR58-TR19 (explained 16% phenotypic variance) and LG 3 between markers TS19-TR56 (explained 23% phenotypic variance) respectively.

Keywords: Chickpea, seed shape classification, seed size index, roundness index, QTL analysis, markers.

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important annual grain legume crop, grown worldwide for human consumption (FAOSTAT 2004). The crop is widely cultivated in the Indian sub-continent, Middle East, Eastern Africa, North America and the Mediterranean region (Cho et al. 2002). The seed is consumed for its high protein content (25.3-28.9%) as well as starch and other essential human nutrients. Two main types of chickpea are recognised, the dark colored, small seeded, angular and fibrous desi type and the beige, large seeded, rams-head shaped and lower fibre kabuli type (McKenzie and Hill 1995). A third, minor type is an intermediate pea type (International Board for Plant Genetic Resources (IBPGR 1993), which is dark or light coloured, small or medium in size and of a rounded shape. Australia has been a producer and major exporter in the international marketplace of both desi and kabuli types since the late 1980s and the crop is recognized as being of relatively high quality with production peaking in 2005 at 187,000 tons. The continued ability of Australian chickpea to attract premium prices is dependent on breeders and growers being able to meet specific market preferences and requirements such as seed size, shape and colour. A number of genetic investigations have been carried out concerning the inheritance of physical seed traits and other important morphological traits in chickpea. Days to flowering is an important trait for crop adaptation and productivity especially when grown under stressed environmental conditions such as late season drought and high temperatures. Kumar and van Rheenen (2000) reported a single gene for flowering time using F_6 derived RILs from the cross of ICCV-2 × JG-62. However, Lichtenzveig et al. (2006) reported two QTLs detected for time to flowering: one in LG1 and another on LG2.

All plant pigments belonging to the class of secondary plant metabolites known as flavonoids (including isoflavones, anthocyanins, flavonols and flavanones). In plants, isoflavones play major roles in the defence responses to pathogen attack (Blount *et al*., 1992; Graham and Graham, 2000), stress tolerance and in establishing the symbiotic relationships between the roots of leguminous plants and rhizobia, which lead to nodulation and nitrogen fixation (Pueppke, 1996). Flavonoids demonstrate a role in protection from ultraviolet (UV) radiation by absorption of UV radiation

Table 1. Comparison of P_1 ICC3996, P_2 S95362 and P445 RILs for physical seed qualities and other morphological traits

Location	Trait	Parental and RILs values mean ± standard error		
		P_1 ICC3996	P_2 S95362	P445 RILs
Horsham	Seed size index round mm (SSI R)	5.93 ± 0.07	7.25 ± 0.18	6.26 ± 0.48
	Seed size index slotted mm (SSI SL)	6.21 ± 0.25	4.73 ± 0.06	5.41± 0.41
	Roundness index mm(RI)	0.80 ± 0.003	0.86 ± 0.003	0.86± 0.03
	Stem colour (where, 1 = green, 5 = red)	5 ± 0.00	1 ± 0.00	1.35 ± 1.06
	Flowering time (where, 1 = early, 9 = late)	4 ± 0.42	7.5 ± 0.57	5.56 ± 1.43
Warne	Seed size index round mm (SSI R)	6.01 ± 0.07	7.37 ± 0.16	6.47 ± 0.48
	Seed size index slotted mm (SSI SL)	4.92 ± 0.07	6.38 ± 0.09	5.67± 0.43
	Roundness index mm (RI)	0.82 ± 0.004	0.87 ± 0.008	0.88± 0.04
	Stem colour (where, 1 = green, 5 = red)	5 ± 0.00	1 ± 0.00	1.38 ± 1.05
	Flowering time (where, 1 = early, 9 = late)	5 ± 0.00	6 ± 0.00	5.84 ± 0.78

and act as insect deterrents and insecticides (Dakora, 1995). Some phytocompounds may be regarded as "accessory health compounds" as they are not essential but may have a significant role in improving human health (Kochian and Gravin, 1999). Muehlbauer and Singh (1987) reported single major gene control of pigmentation of flowers, stems and seeds. Shape is also an important seed quality measure that directly affects the seed appearance and uniformity and is often used as a quality indicator to importers and consumers. However, the market drivers for certain shape characters are influenced greatly by cultural preference. For example the pea type is popular in central India (Meena et al. 2004), the smaller desi type is mainly imported by India and surrounding countries and consumers in North and South America, Europe, the Middle East and Africa prefer the kabuli type. Therefore, in order to produce seed of a specific shape, to meet a specific market demand, knowledge of the genetics that determine shape, together with a method to precisely measure and discriminate among the possible shape variants is required. Several methods are used to measure seed shape characteristics. Of these, the low cost traditional visual assessments of seed appearance have mainly been used to determine segregation for seed shape among germplasm (Meena et al. 2004). Although Saxena and Singh (1987) documented seed-shape descriptors including anatomical structure of various seed types of chickpea, no absolute definitive description for the desi, kabuli or pea shape was proposed. For soybean, Nelson and Wang (1989) proposed a visual scoring system to describe a broad variation of seed shape. In general, classification of shape based on visual inspection alone is highly subjective. Although skilled and experienced inspectors may be consistent in their own visual assessments, disagreement occurs among inspectors is in part due to the vague boundaries of the assessment criteria. Alternatively, image analysis systems were developed for classification of wheat varieties in the USA, UK, Canada and Australia (for review see (Sapirstein 1995). Sakai et al. (1996) investigated two-dimensional image analysis for rice grain shape. Image analysis is fast, non-destructive, offers the potential to measure novel characters and enables electronic data storage; however, there are also limitations in identifying heterogeneous characters within a single variety (Sapirstein 1995). It is expensive to set up and requires skilled professionals for operation and data analyses. To date, limited studies have been performed to classify chickpea seed on the basis of shape (Meena et al. 2004). Thus, a novel method, which is reliable, cost effective, user friendly, easily portable and has the potential for wide acceptance, is highly desirable. In order to accurately select and breed for a desirable seed shape, an understanding of the number of genes underpinning the trait and their mode(s) of inheritance is required along with an understanding of the potential of environment to impact on genetics of seed shape. To determine these parameters, the mitigating genetic effects of morphological factors should be considered such as the seed width, length and seed surface texture along with environmental influence. More and D'Cruz (1970) suggested two dominant genes *Rsa* and *Rsb,* for rough testa. Recently, Meena et al. (2004) investigated the genetics of seed shape in F_2 populations of three different chickpea crosses without documenting any classification descriptor. They also reported that the inheritance mechanism for seed shape is under digenic control. The aims of the current study were to: (1) Develop a selection tool for chickpea seed shape, (2) Determine the Mendelian inheritance mechanism for chickpea seed shape, to provide fundamental and practical breeding knowledge and (3) find molecular markers associated with major QTL for seed shape, stem colour and flowering time that may be targeted for future selective breeding.

Material and Methods

Plant materials

Seed from two field trials (CQHO06 and CQMC06) were used in this study which comprised F_5 derived F_7 ($F_{5:7}$) Recombinant Inbred Line (RIL) populations that were produced from intraspecific crosses of a kabuli-type (S953-62) with two desi-types (*cv*. Howzat and ICC3996) at the Department of Primary Industries (DPI) in Horsham, Victoria, Australia. The populations were labeled P445 (ICC3996 X S95362) and P453 (S95362 X *cv*. Howzat) comprising 91 and 105 lines, respectively. RILs from each population, together with parental genotypes, were grown as duplicated and randomized single line plots (35 plants per plot) at two environmental sites following special row column design to maximize the chance for getting maximum variation between lines within each environmental sites, where the environments had contrasting rainfall, soil type and temperature during grain fill over the 2005 growing season.

Table 2. Phenotypic correlation coefficient between physical seed quality traits and other morphological traits of the two recombinant inbred line populations at two different locations

Population	Site	Trait	SSIR	SSISL	RI	St Cl	FT
P445	Horsham	SSIR	1.00				
		SSISL	0.86				
		RI	-0.25	0.26			
		St Cl	0.10	0.04	-0.10		
		FT	-0.03	-0.02	0.01	-0.22	1.00
		FT					
P445	Warne	SSIR	1.00				
		SSISL	0.85				
		RI	-0.24	0.30			
		St Cl	0.03	-0.03	-0.14		
		FT	-0.07	-0.06	0.02	-0.12	1.00
P453	Horsham	SSIR	1.00				
		SSISL	0.86				
		RI	-0.21	0.30			
		St Cl	0.05	-0.03	-0.17		
		FT	0.05	0.13	0.15	-0.02	1.00
P453	Warne	SSIR					
		SSISL	0.87				
		RI	-0.10	0.38			
		St Cl	0.03	-0.10	-0.27		
		FT	0.00	0.05	0.11	0.03	1.00

Where: SSIR = seed size index round, SSISL = seed size index slotted, RI = roundness index, St. Cl = stem colour, FT = flowering

Site 1 was located in a dryland paddock at the Plant Breeding Centre, Horsham, Victoria, Australia (Horsham, longitude 142.16º E, latitude 36.71º S, 143 m elevation) and Site 2 was located at Warne, Victoria, Australia (longitude 143.03º E, latitude 35.79º S, 98 m elevation).

Data collection

Each RIL and parental genotype was phenotyped for stem colour using a standard scale used by the chickpea breeding program at Department of Primary Industries, Horsham (personal communication with chickpea breeder Kristy Hobson), flowering time (where, 1 = early and 9 = late. (personal communication with Kristy Hobson). Following harvest, raw seed shape data were collected in two ways; with calipers and with nested sieves.

Caliper method

Digital calipers (AMRAD Tools, model CE15) were used to collect the parental reference data for seed length, width and height. 100 seeds were drawn randomly from each parental type for the measurement. In all cases, seed length (including beak) was the longest axis. The calipers were not subsequently used to collect RIL data due to processing speed and accuracy limitations.

Sieving method

The RIL and parental seed from each plot were divided randomly into two sub-samples, with up to 250 g in each, and were scored for mean seed shape indices as explained below:

Seed size index - round sieves (SSI_R)

Seed size was determined according to the Australian Pulse Quality Laboratory Manual, (Method No. APQ-103). This involves sieving 250 g of a seed sample through a nested set of seven sieves with decreasing circular holed diameters, ranging from 4 mm to 10 mm. The weighted mean weight of seed retained on each of the sieves is then calculated to provide the seed size index (SSI_R). This method categorizes seed shape on seed size based solely on the longest dimension of the seed cross section through the axis.

Seed size index - slotted sieves (SSI_{SL})

A modified version of the SSI_R method was developed in this study that employs another set of seven sieves with 2.5 cm long slotted holes and decreasing gap widths of 10 mm to 4 mm. The weighted mean weight of seed retained on each of the sieves is then calculated to provide the seed size index (SSI_{SL}). This method categorizes seed shape on seed size based solely on the shortest dimension of the seed cross section through the axis.

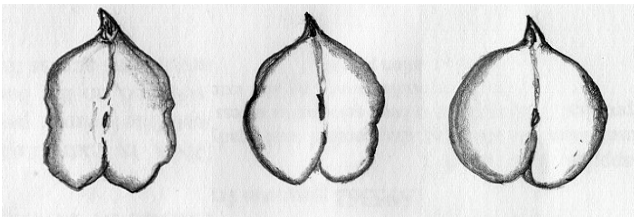

Fig 1. A graphical representation of seed shape morphology with varying roundness indices (RI) provided in bold

A novel method for seed shape classification in chickpea

In order to gain a multi-dimensional (axial) measure of seed shape, the ratio of seed size index slotted (SSI_{SL})/ seed size index round (SSI_R) was determined for each sample and denoted the 'Roundness Index' (RI). Those seed with a RI value close to 1.0 possessed a circular cross section, whereas those with lower RI values possessed a more angular (out-of-round) cross section (Fig. 1).

Roundness index (RI) was calculated for a measure of seed shape as: Seed size index slotted (SSI_{SL}) / Seed size index round (SSI_R)

Visual observation

Seed were also visually scored to investigate the Mendelian segregation ratios among the populations between the two environmental sites and classified into three distinct classes as desi, kabuli or pea shape by considering the fine topological details (including sharp ridges, grooves, roughness and cross-sectional circularity) of the seed surface.

Statistical analysis

Analyses of variance (ANOVA) using GENSTAT ver. 4.2 was employed to analyze phenotypic variability (σ^2_P) for Roundness index (RI) of both RIL populations. A General linear Model (GLM) was used to estimate variance components for genetic parameters of RIs including genetic variance (σ^2_G), environmental variance (σ^2_E) and genetic *vs* environmental interaction (σ^2_{G*E}) using MINITAB® (Release 14.13). The heritability (h^2) for each population was estimated according to Nyquist (1991) using the standard formula of ratio of genotypic variance (σ^2_G) to the phenotypic variance (σ^2_P). Where, phenotypic variance was partitioned into genetic variance (σ^2_G), genetic *vs* environmental variance (σ^2_{G*E}) and environmental variance (σ^2_E). Thus, heritability was calculated as: $h^2 = \sigma^2_G / \sigma^2_P = \sigma^2_G / \sigma^2_G + \sigma^2_{G*E} + \sigma^2_E$
The goodness-of-fit to expected segregation ratios for various inheritance models were determined by Chi-square (χc^2) analysis with the threshold χc^2 value 0.05.

Quantitative trait loci (QTL) analysis

A framework chickpea linkage map was developed from a Recombinant Inbred Line (RIL) progenies (P445) derived from an intraspecific cross between the desi cultivar ICC3996 and the kabuli cultivar S95362 to test the association between markers for seed shape, stem colour and flowering time. This particular cross was also previously used to investigate the Mendelian inheritance mechanism for seed shape. A total of 80 SSR markers previously characterised by Winter *el al*. (1999) and Lichtenzveig *et al*. (2005) were used that were polymorphic between the parental genotypes. PCR reactions of 12.5 µl were performed in a Eppendorf Mastercycler and comprised PCR buffer (Bioline, Australia), 0.2 mM of combined dNTPs (Bioline, Australia), 2.5 mM $MgCl_2$ (Bioline, Australia), 1 µM of each primer (Sigma-Genosys, Australia), 1 unit of *Taq* polymerase (Bioline, Australia) and 10 ng of template DNA. The PCR reaction was an initial denaturation step at 94 °C for 3 min followed by 35 cycles of 94 ºC for 30 s, 50 ºC - 60 ºC (as appropriate for the primer pair) for 1 min and 72 ºC for 90 s, with a final elongation step at 72 ºC for 5 min. Amplified SSR markers were separated by electrophoresis in TBE buffer on 1.4% agarose gel, stained and visualized. Clear and single locus-specific markers were then scored as either allele 1 (parent 1) or allele 2 (parent 2).

Each segregating marker was tested for goodness of fit to the expected Mendelian segregation ratio of 1:1, representative of a co-dominantly inherited single locus in an RIL $F_{5:7}$ population using Chi-square (χc^2) analysis (P <0.05). Linkage analysis was performed on the non-distorted markers using Map Manager QTX Ver. 0.23 (Manly *et al*. 2001). Linkage groups (LGs) were established by assigning markers with the "make linkage groups" command at P = 0.0001 (equivalent to a LOD score of 3.0). The marker order of each linkage group was verified using the "ripple" command. Final map distances were calculated by applying the 'Kosambi' function (Kosambi, 1944). LG labeling was done according to the anchoring of SSR markers from previously developed maps (Winter *et al*., 2000). Quantitative values were employed to perform QTL detection for seed shape, stem colour and flowering time. QTL analysis was carried out using MapManager QTX software version 0.23 (Manly *et al.,* 2001). Two different methods were used to identify QTL. Firstly, single point analysis was performed on all markers to determine those significantly associated with seed shape, stem colour and flowering time using the 'Links Report' command. Regions of high marker association were proposed to restrain a QTL effect that was expressed as the percentage of total variance, calculated from the trait variance and residual variance. The significance of each association between marker and QTL was determined by a likelihood ratio statistics (LRS) value. Secondly, simple interval mapping (SIM) at 1 cM was appl- ied to identify putative QTL for traits assessed among the two different environments (Horsham and Warne). Although, in recent time composite interval mapping (CIM) has been considered to be more powerful than simple interval mapping (SIM) it

Table 3. Estimate of genotypic and environmental variance components and heritability in the broad sense for seed shape (RI) within the P445 and P453 RIL populations.

Genotype	Source of variance	Estimated value	%
RIL P445	Site	0.00007	4.5
	Replicate(Site)	0.00004	2.5
	Genotype	0.00127	81.9
	Site* Genotype	0.00011	7.0
	Error	0.00006	3.8
	Total	0.00155	100
	Heritability	0.875	87.5
RIL P453	Site	0.00002	1.7
	Replicate(Site)	0.00003	2.6
	Genotype	0.00093	83.0
	Site* Genotype	0.0001	8.9
	Error	0.00004	3.5
	Total	0.00112	100
	Heritability	0.885	88.5

does have some disadvantages. For example, CIM uses background markers which can absorb effects of target QTL and also the permutation test is slow in CIM and may not be appropriate due to removal of the association of QTL with background markers. On the other hand SIM takes proper account of missing data, allows examinations of positions between markers and also give improved estimates of QTL effect.) In addition the current map was not dense enough to use CIM, thus SIM was more suitable approach for the current map density. The significance (effect) of QTL was determined empirically using Churchill and Doerge's (1994) permutation test with 1000 replications, which gave a LRS/LOD [logarithm (base 10) of odds] value for the size of the QTL and a ~ 95% confidence indication on the location of the QTL within the marker intervals.

Results and discussion

Inheritance of seed shape in chickpea

The ANOVA indicated significant differences in variation in seed shape between lines within populations based on the RI ($P \leq 0.001$). The distribution of shape for both populations at both sites appeared to be continuous when assessed using RI (Fig. 2), and suggested that seed shape was inherited in a quantitative manner in the genotypes assessed. Several RILs having a RI higher than the respective rounder S95362 (mean RI 0.86 ± 0.003) parental type (Table. 1) (Fig. 2) in both populations, indicated that alleles governing the roundness trait were contributed from both parents and were expressed additively in the RIL populations. However, the distribution of RI *vs* visual scoring data overlapped (Fig. 3), and suggested that the RI values were not discriminative enough to statistically classify or create a cut-off between the RILs into the three previously described seed shape classes (kabuli, desi and pea). There is a noted overlap between RI values and visual shape groups (Fig. 3), however, by using the parental mean RI and standard deviation a general RI for desi shape (0.81 ± 0.014) would be 0.79 - 0.82 mm and for kabuli shape (0.86 ± 0.007) would be 0.85 - 0.86 mm. However, due to the overlap and inability to draw solid separation between progeny phenotype classes based on RI, the visual shape data which included the assessment of the seed's fine topological details was employed for segregation analysis.

Phenotypic correlation coefficients among observed traits for each population and location at $P \leq 0.01$ revealed some meaningful relationship between traits (Table 2). Skinner *et al*. (1999) suggested that the correlation coefficients greater than 0.71 or smaller than -0.71 are biologically meaningful. In this present study positive correlation coefficients were observed between traits SSIR – SSISL and RI - SSISL. Negative correlation was also observed between RI - SSIR, St Cl – RI and St Cl – FT in both P445 and P453 across two environmental sites (Table 2).

Genetic variance for RIs was estimated as 81.9% in the P445 and as 83% in the P453 population of the total variation (Table 3). Analysis of genotype by site interaction explained 7% and 8.9% of total variation for the P445 and P453 populations, respectively (Table 3). This indicated that environmental effect had a relatively low influence on seed shape in these populations. Heritability estimates for RI values were also determined using combined data from both locations, and were calculated as 0.87 (87% of total variation) for the P445 and as 0.88 (88% of total variation) for the P453 population (Table 3). The high magnitude of heritability also indicated that the environmental influences were less on the trait of interest, in this instance seed shape.

The data from visual scoring was used to investigate the Mendelian segregation ratios among the different seed shapes within the populations between the two environmental sites. The observed seed shape segregation ratios from visual scoring data for the P445 population among sites was 30 (desi shape):19 (kabuli shape):42 (pea shape) (Warne) and 32 (desi shape):18 (kabuli shape):41 (pea shape) (Horsham). These both best fit the expected ratio of 1 (desi):1 (kabuli):2 (pea) for a two gene model (Table 4). The observed seed shape segregation ratios for the P453 population among sites were 24 (desi shape):47 (kabuli shape):34 (pea shape) (Warne) and 24 (desi shape):50 (kabuli shape):31 (pea shape) (Horsham). These also best fit a two gene model with an expected 1:2:1 ratio (Table 4). The segregation ratios among the three shape groups did not significantly fit any other expected gene ratios tested for either population (Table 4). However, the observed continuous distributions indicated there may also be gene modifiers with minor effects influencing seed shape (Fig. 3). Even so, the results indicated that desi and kabuli shapes were differentiated by two major genetic factors. This supports the findings of earlier studies by Kumar *et al*. (1985) and Meena et al. (2004) who reported similar genetics conditioning seed shape in desi x kabuli crosses. However, Kumar et al. (1985) observed a contrasting behavior of different seed shapes in two different sets of desi x kabuli crosses and reported that the kabuli parent influenced the segregation of different seed shapes in those crosses. Knights (1980) and Hawtin and Singh (1980) also conducted similar experiments with chickpea crosses and presented results as a percentage of difference in seed shape

Table 4. F5 derived F7 RILs segregation for seed type (desi, kabuli and pea) based on visual scoring in desi vs kabuli chickpea crosses and Chi-square (χc^2) fit to genetic model for seed shape

Site	Genotype	No. of Lines	$F_{5:7}$ generation			df	Tested ratio	χc^2	P value
	RIL P445		Desi	Kabuli	Pea				
Warne	CQMC05	91	30	19	42	2	1 : 1 : 2	3.198	0.202 ^{ns}
						2	1 : 2 : 1	34.834	0.000
						2	2 : 1 : 1	22.725	0.000
Horsham	CQHO05	91	32	18	41	2	1 : 1 : 2	5.356	0.068 ^{ns}
						2	1 : 2 : 1	35.843	0.000
						2	2 : 1 : 1	20.117	0.000
	RIL P453								
Warne	CQMC05	105	24	47	34	2	1 : 1 : 2	23.114	0.000
						2	1 : 2 : 1	3.057	0.216 ^{ns}
						2	2 : 1 : 1	34.162	0.000
Horsham	CQHO05	105	24	50	31	2	1 : 1 : 2	30.486	0.000
						2	1 : 2 : 1	1.171	0.556 ^{ns}
						2	2 : 1 : 1	37.189	0.000

* ns = non significant

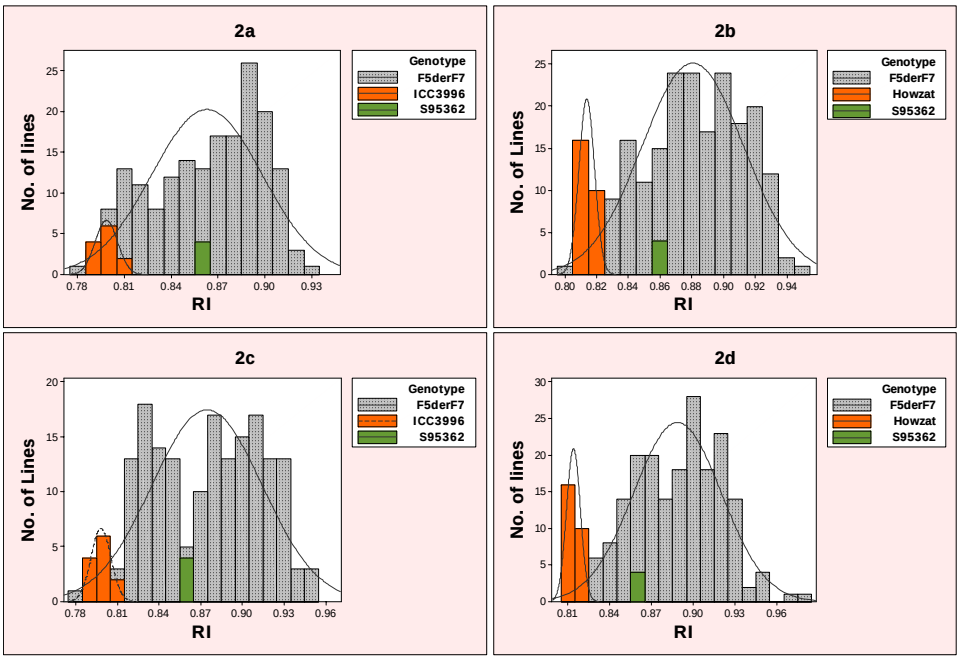

Fig 2. Distribution of seed shape among respective parental and RIL of the P445 and P453 populations at Horsham (2a and 2b, respectively) and Warne (2c and 2d, respectively) based on RI.

without any conclusion regarding the mode of inheritance conditioning the shape.

QTL analysis for seed shape and other agronomic traits

An intraspecific linkage map was constructed with 80 SSR markers distributed over 10 linkage groups covering approximately 204.4 cM of the chickpea genome at an average marker density of 2.8 cM. At a LOD-score of 3.0, seven (8.7%) markers remained unlinked. LG 6 represented the largest linkage group, comprising 11 markers and spanning 70.4 cM. Naming of the LG was in accordance with presence of the anchor markers from the most extensive chickpea genome map of Winter *et al*. (2000). The present map is a sparse frame work but revealed similar marker distribution among LG with other published chickpea genetic maps reported by Winter *el al*. (1999) and Winter *et al*. (2000) although they were developed using different mapping populations and marker sets. Seven linkage groups showed sensible consistency between the current chickpea map and the linkage maps reported by Winter el al. (1999) and Winter et al. (2000). Markers on LG 7 were collinear with markers on LG 5 $_{W99}$ Winter et al. (1999) and LG 7 $_{W00}$ Winter et al. (2000) of the both interspecific linkage map. Markers that

Table 5. Significant associations between molecular markers and putative QTL for seed shape and other morphological traits in the $F_{5:7}$ population (ICC3996 x S95362) at two different sites, detected by single point analysis

Site	QTL name	Linkage group	Marker	[1]LRS	[2]Variance(σ) %	P	[3]Additive effect
Horsham	RI H	LG 2	TA110	6.9	5	0.008	-0.01
		LG 2	TA27	8.2	6	0.004	-0.01
		LG 2	H6D11	7.2	5	0.007	-0.01
		LG 2	H4BO9	7.3	5	0.006	-0.01
		LG 2	H1AO6	10.7	9	0.001	-0.01
Warne	RI W	LG 2	TA110	11.4	9	0.000	-0.01
		LG 2	TA27	6.4	5	0.011	-0.01
		LG 2	H6D11	4.9	4	0.026	-0.01
		LG 2	H4BO9	5.3	4	0.021	-0.01
		LG 2	H1AO6	8.9	7	0.002	-0.01
Horsham	St. colour H	LG 2	TA194	6.1	8	0.013	0.35
		LG 2	TR58	10.8	9	0.001	0.34
		LG 2	TR19	6.7	5	0.009	0.29
	F. time H	LG 3	TS19	26.1	21	0.000	-0.68
		LG 3	STMS14	9.6	7	0.001	-0.42
		LG 3	H1BO4	7.4	6	0.006	-0.38
		LG 3	H3FO9	10.1	8	0.001	-0.43
		LG 3	H3FO8	15.4	12	0.000	-0.52
Warne	St. colour W	LG 2	TA194	8.4	12	0.003	0.43
		LG 2	TR58	16.1	14	0.000	0.41
		LG 2	TR19	11.2	10	0.000	0.37
	F. time W	LG 3	TS19	29.0	23	0.000	-0.39
		LG 3	STMS14	10.4	8	0.001	-0.23
		LG 3	H1BO4	7.2	6	0.007	-0.20
		LG 3	H3FO8	10.8	8	0.001	-0.24
		LG 3	H4GO7	6.7	8	0.009	-0.24

[1]LRS = likelihood ratio statistic for association of the trait with this locus.
[2]Variance (σ) % = the amount of total variance, which would be explained by a QTL at this locus.
[3]Additive effect = the additive regression coefficient for the association.
Where: RI = roundness index, St. colour = stem colour, F. time = flowering time and H = Horsham (an environmental location) and W = Warne (an environmental location).

Table 6. Putative QTL for seed shape and other morphological traits identified using simple interval mapping

Trait [1]	Marker interval	QTL name	LG	LRS/LOD	Variance(σ) %
Seed shape					
RI W	TA110 – TA27	QTL_1 RI W	2	11.6/2.5	9
Stem colour					
St. colour H	TR58 – TR19	QTL_1 St. Cl H	2	10.8/2.3	9
St. colour W	TR58 – TR19	QTL_1 St. Cl W	2	16.4/3.5	14
Flowering time					
F. time H	TS19 – TR56	QTL_1 F.T H	3	26.1/5.6	21
F. time W	TS19 – TR56	QTL_1 F.T W	3	29.0/6.2	23

[1]*key for this table as per previous table*

were located on LG 1 of the current map were collinear without inversion with the same markers on LG 6 $_{W99}$ and LG 1 $_{W00}$. LG 2, LG 3, LG 4, LG 5 and LG 6 also represent the same markers of previous chickpea maps with minor inversion. Roundness index (RI) was used as a measure of seed shape in this study. Using single point analysis one marker from each environment (H1AO6 and TA110) was found associated with RI [RI H(Horsham) and RI W(Warne)] , at a LRS threshold of 9.2 (equivalent to LOD score 2) and was located on LG 2 (Table 5). Four markers from each location positioned on LG 2 were below the LRS threshold; however, the *P* values were < 0.05, suggesting that these four markers may be significantly associated with RI (Table 5) The negative additive regression coefficient for the marker associated to the QTL suggested that the marker might be associated with the angular seed shape. Single point analysis revealed marker TS19 as being associated with flowering time (F. Time H and F. Time W) on LG 3 at a LRS threshold of 13.8. Four more markers, identified in both environments and positioned on LG 3, fell short of the LRS threshold, but the *P* values ($P < 0.05$) suggested that they may also have significant association with flowering time (Table 5). Single point analysis also detected three markers on LG 2, which fell short of the LRS threshold, but again the *P* values < 0.05,

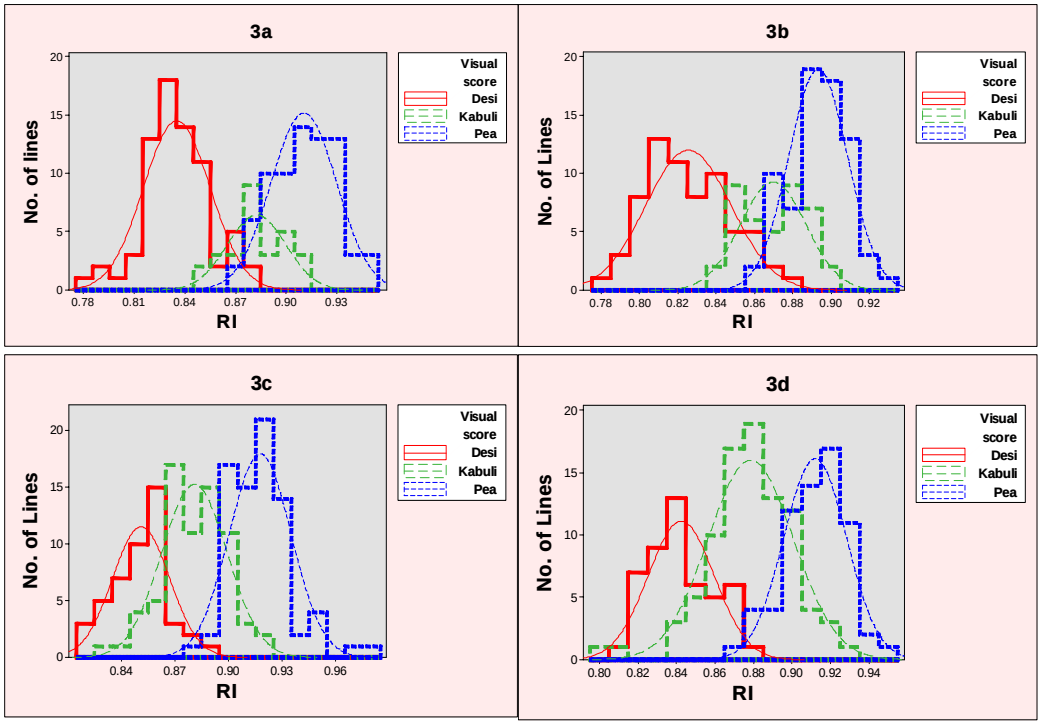

Fig 3. Distributions of seed shape based on RI and visual score within the P445 3a (Horsham), 3b (Warne) and P453 3c (Horsham), 3d (Warne) RIL populations

suggested that these markers may have significant association with stem colour (Table 5).

Simple interval mapping identified one putative region for a QTL that was significantly associated with seed shape (RI) on LG 2 between markers TA110 and TA27 in one environment (Warne) with an LRS value of 11.6 (9.2 < LRS < 13.8). The QTL explained 9% of the total phenotypic variation (Table 6; Figure 4). A similar result was also identified by single point analysis. One putative QTL for stem colour was identified by simple interval mapping on LG 2 between markers TR58 and TR19 at an average LRS value of 13.6 (9.2 < LRS < 13.8) and explained up to 14% of the total phenotypic variation (Table 6; Figure 4). Simple interval mapping also identified one major QTL that was significantly associated with flowering time on LG 3, between markers TS19 and TR56 with an average LRS value of 27.5 that accounted for 23% of the phenotypic variation (Table 6; Figure 4).

Only one putative QTL for roundness index that represents a quantitative measure of seed shape was identified on LG 2 between markers TA110 and TA27 with an LRS value of 11.6 that explained 9% of the phenotypic variation. However, Mendelian inheritance revealed seed shape was to be controlled by two major genetic factors. The low phenotypic contribution explained by the QTL may be an indication of the possible involvement of additional unidentified loci, which have not been detected either because of incomplete genome coverage or the small RIL population size (Dholakia et al., 2003). Therefore, future investigations to detect additionnal and larger QTL for seed shape are required. A QTL for stem colour was also detected on LG 2 between markers TR58–TR19 with a LRS value of 16.4 that explained 16% of the phenotypic variation. However, Cho *et al*. (2002) mapped the major pigmentation gene *'c'* to LG 8 13.5 cM apart from a STMS marker Tr33. A QTL for flowering time was identified on LG 3 between markers TS19 and TR56 with a LRS value of 29.0 that explained 23% of the phenotypic variation. This is in agreement with the findings of Cho *et al*. (2002) who also identified a QTL for flowering time on LG 3. Cho *et a.l* (2002) detected their QTL between markers TS57 and TA127. However, these markers were not polymorphic in this present map and hence the exact chromosomal location could not be compared. In another study, Cobos *et al*. (2007) detected a QTL for days to flowering on LG 4 closely linked to markers STMS and GAA47 and most recently, Cobos *et al*. (2009) mapped a QTL for flowering time on LG 3 closely linked to marker TA142. The difference in the method of phenotypic assessment of flowering time may account for the different QTL detected.

Applicability of RI for definitive seed shape classification

Although the RI was applied as a measure of seed shape in this study, caution must be taken when using this value for classification of seed into one of the predetermined shape descriptors of kabuli, desi or pea. The overlap among the distributions of RIs *vs* visual scoring data indicated that the RI does not account for the finer structure at the seed surface, and therefore is not an optimal shape classification tool. Indeed, Saxena and Singh (1987) previously reported that seed topographical features, such as sharp ridges, grooves and roughness, were highly correlated with the predetermined

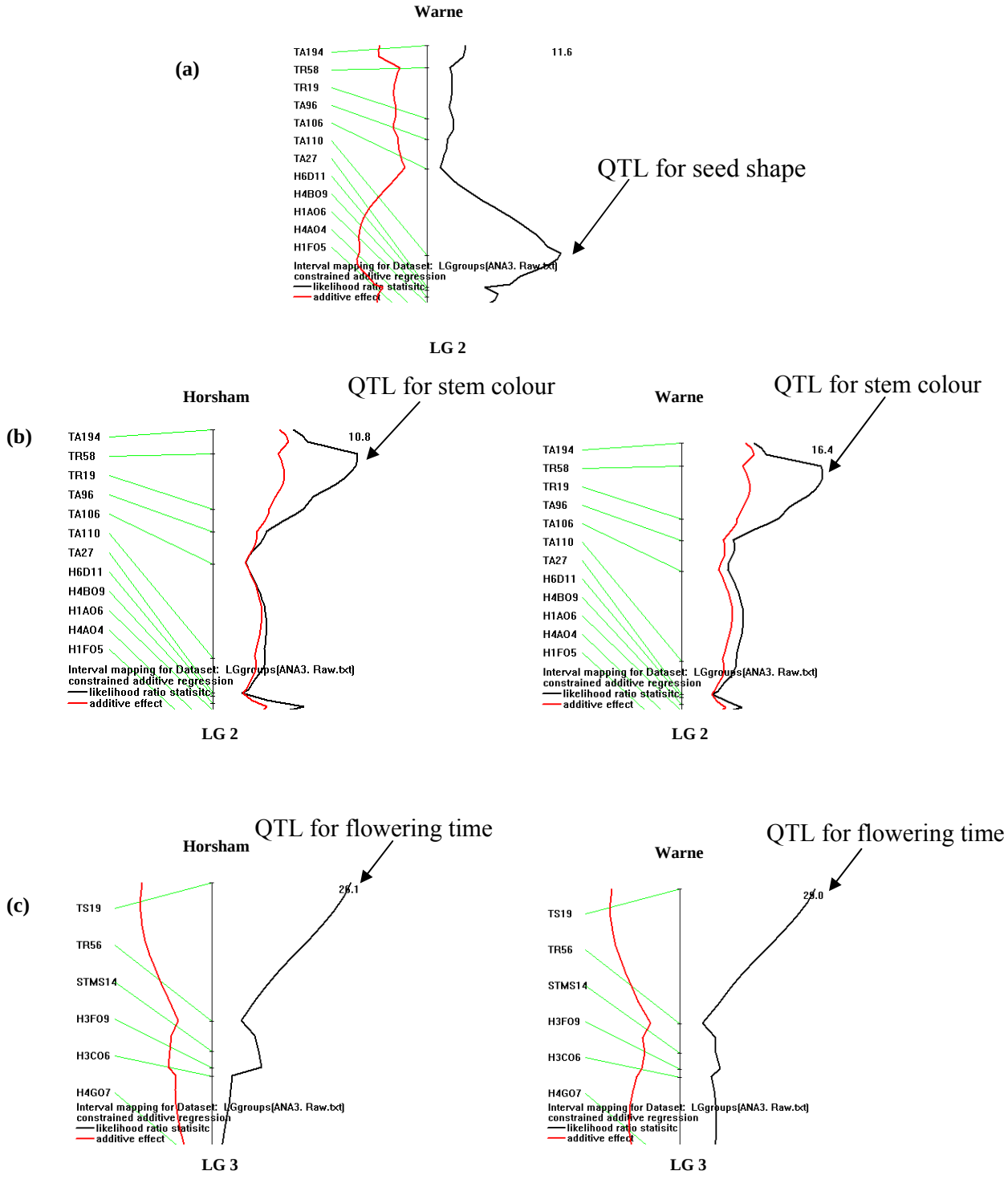

Fig 4. Putative QTL detected for seed shape (a) at one site; Warne, stem colour (b) and flowering time (c) in two different environmental sites; Horsham and Warne

visual seed shapes (desi, kabuli and pea). However, RI is a reproducible quantitative measure of seed cross-sections and may have potential application in breeding program as a selection tool for bold seed types. For example, in a desi x desi cross or kabuli x kabuli cross breeding program, it is not important for a breeder to select a variety on the basis of being a desi shape or a kabuli shape, rather how bold or plump the seed is. The major rationale for QTL mapping is to identify genomic regions conditioning important agronomic traits, particularly for traits that are difficult or expensive to phenotype and may be affected by environmental conditions. In the present investigation, QTL for several of most important agronomic traits in chickpea were identified. Furthermore, the molecular markers that revealed close association with the traits in this study may target for selective breeding in future breeding programs.

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References

Blount JW, Dixon RA, Paiva NL (1992) Stress response in alfalfa (*Medicago sativa* L.). Physiol Mol Plant Pathol 41: 333-349.

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138: 963-971.

Cho S, Kumar J, Shultz J, Anupama K, Tefera F, Muehlbauer F (2002) Mapping genes for double podding and other morphological traits in chickpea. Euphytica 128: 285-292.

Cobos MJ, Rubio J, Fernandez-Romero MD, Garza R, Moreno MT, Millan T, Gil J (2007) Genetic analysis of seed size, yield and days to flowering in a chickpea recombinent inbred line population derived from a kabuli x desi cross. Ann Appl Biol 151: 33-42.

Cobos MJ, Winter P, Kharrat M, Cubero JI, Gil J, Millan T, Rubio J (2009) Genetic analysis of agronomic traits in a wide cross of chickpea. Field Crop Res 111(1/2): 130-136.

Dakora FD (1995) Plant flavonoids: biological molecules for useful exploitation. Aust J Plant Physiol 22: 87-99.

Dholakia BB, Ammiraju JSS, Singh H, Lagu MD, Roder MS, Rao VS, Dhaliwal HS, Ranjekar PK, Gupta VS (2003) Molecular marker analysis of kernel size and shape in bread wheat. Plant Breed 122: 392-395.

Faostat-Agriculture (2004). Database. http://www.fao.org /waicent/portal/statiistic.es.asp.

Graham TL, Graham MY (2000) Defense potentiation and competency: redox conditioning effects of salicylic acid and genistein. In: Keen N, Stacey G (ed) Plant microbe interactions, Vol. 5 APS Press, St. Paul.

Hawtin GC, Singh KB (1980) Kabuli-desi introgression problems and prospects. In: Workshop on chickpea improvement. ICRISAT, Hayderabad, India pp. 51-80. (In proc. Intern.)

IBPGR (1993) Descriptors for Chickpea (*Cicer arietinum* L.). Rome, Italy.

Knights EJ (1980) Kabuli-desi introgression. The experience in Australia. In: Workshop on chickpea improvement. ICRISAT, Hyderabad, India pp 70-74. (In Proc.Intern)

Kochian L, Garvin D (1999) Agricultural approaches to improving phytonutient content in plants: An overview. Nutr Rev 57: 813-818.

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12: 172- 175.

Kumar J, Bhal PN, Raju DB (1985) Seed type variation in desi-kabuli crosses of chickpea. Intern Chickpea Newsletter 1-2.

Kumar J, Rheenen HA (2000) A major gene for time of flowering in chickpea. J Hered 91: 67–68.

Lichtenzveig J, Bonfil JD , Zhang HB, Shtienberg D, Abbo S (2006) Mapping quantitative trait loci in chickpea associated with time to Xowering and resistance to *Didymella rabiei* the causal agent of Ascochyta blight Theor Appl Genet 113: 1357-1369.

Lichtenzveig J, Scheuring C, Dodge J, Abbo S, Zhang HB (2005) Construction of BAC and BIBAC libraries and their applications for generation of SSR markers for genome analysis of chickpea, Cicer arietinum L. Theor Appl Genet 110: 492-510.

Manly KF, Cudmore H, Robert JR, Meer JM (2001) MapManager QTX, cross-platform software for genetic mapping. Mamm Genome 12: 930-932.

McKenzie BA, Hill GD (1995) Growth and yield of two chickpea (*Cicer arietinum* L.) varieties in Canterbury,New Zealand. New Zealand J Agric Sci 23: 467-474.

Meena HS, Kumar J, Yadav SS (2004) Genetics of seed shape in chickpea (*Cicer arietinum* L.). Ann.Agric.Res.New Series 25: 439-441.

More DC, D'Cruz R (1970) Genetic studies in Bengal gram (*Cicer arietinum* L.). Poona Agricultural College Magazine 60: 27-32.

Muehlbauer FJ, Singh KB (1987) Genetics of Chickpea. In: M.C. Saxena & K.B. Singh (ed), The Chickpea, CAB International pp 99–126..

Nelson RL, Wang P (1989) Variation and evaluation of seed shape in soybean. Crop Sci. 29: 147- 150.

Nyquist WE (1991). Estimation of heritability and prediction of selection response in plant populations. Crit Rev Plant Sci 10: 235-322.

Pueppke JL (1996) The genetics and biochemical basis for nodulation of legumes by rhizobia. Crit Rev Biotechnol 16: 1-51.

Sakai N, Yonekawa S, Matsuzaki A, Morishima H (1996) Two dimensional image analysis of the shape of rice and its application to seperating varieties. J Food Eng 27: 397-407.

Sapirstein HD (1995) Varietal identification by digital image analysis. In: American Association of Cereal Chemists Inc, St. Paul.

Saxena MC, Singh KB (1987) The Chickpea. In: CAB International, Aleppo, Syria.

Singh H, Ekbote RB (1936) The inheritance of seed characters in gram (*Cicer arietinum* L.). Indian J. of Agric Sci 6: 1087-1104.

Skinner DZ, Bauchan GR, Auricht G and Hughes S (1999) A method for the efficient management and utilization of large germplasm collections. Crop Sci 39: 1237-1242.

Winter P, Benko-lseppon HB, Ratnaparkhe M, Tullu M, Sonnante G, Pfaff T, Tekeoglu M, Santra D, Sant VJ, Rajesh PN, Kahl G, Muehlbauer FJ (2000). A linkage map of chickpea (Cicer arietinum L.) genome based on recombinent inbred lines from *c. arietinum* x *c. reticulatum* cross: localization of resistance genes for Fusarium wilt races 4 and 5. Theor Appl Genet 101: 1155-1163.

Winter P, Pfaff T, Udupa SM, Hüttel B, Sharma PC, Sahi S , Arreguin-Espinoza R, Weigand F, Muehlbauer FJ, Kahl G (1999) Characterization and mapping of sequence-tagged microsatellite sites in the chickpea (*Cicer arietinum* L.) genome. Mol Genet 262: 90-101.