Genome Size and Seed Mass Analyses in *Cicer arietinum* (Chickpea) and Wild *Cicer* Species

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Abstract. The genome size of cultivated *Cicer arietinum* and 12 wild *Cicer* sp. including seven annual and five perennial species were analyzed using flow cytometry. A significant 2C genome size variation was observed among the *Cicer* sp. The 2C genome size ranged from 1.00 pg in wild species, *Cicer judaicum*, to 1.76 pg in cultivated species, *C. arietinum*. The wild perennial species all had a genome size of ≈ 1.6 pg. Most if not all of this genome size variation occurred among wild annual species. A significant positive correlation between 2C genome size and seed mass was observed among 12 wild *Cicer* sp. at $\alpha = 0.05$. However, artificial selection appears to decrease nucleotide effects in cultivated *C. arietinum*, which resulted in no correlation between seed mass and genome size at $\alpha = 0.05$.

The genus *Cicer* belongs to family Leguminosae, subfamily Papilionaceae, tribe Cicerereae, and is well known for the cultivated taxon, *C. arietinum* or chickpea. *Cicer arietinum* is the second most widely grown annual legume crop in the world, and has been mainly cultivated in the Indian subcontinent, West Asia, North Africa, America, and Australia (Singh et al., 2008). Besides the cultivated species, this genus is known to have 43 wild species including 8 annual and 35 perennial species (Singh et al., 2008, 2014; van der Maesen, 1987). The wild *Cicer* sp. are commonly found in West Asia and North Africa covering Turkey in the north to Ethiopia in the south, and Pakistan in the east to Morocco in the west (Singh et al., 2008). The wild annual species such as *Cicer reticulatum*, *Cicer echinospermum*, *Cicer pinnatifidum*, *C. judaicum*, *Cicer bijugum*, and *Cicer cuneatum* are being used in breeding programs to expand chickpea genetic diversity (Ladizinsky and Adler, 1976; Pundir and Mengesha, 1995; Pundir and van der Maesen, 1983; Singh and Ocampo, 1993; van der Maesen, 1980).

Knowledge of genetic relationships between the cultivated *C. arietinum* and its wild relatives plays an important role in assessing the origin of *C. arietinum* and in using its close relatives to facilitate the transfer of agronomically useful

traits into cultivated species (Singh et al., 2008). Karyotype studies in these *Cicer* sp. have been well documented (Galasso et al., 1996; Ladizinsky and Adler, 1976; Ohri and Pal, 1991) and showed that annual and perennial species consistently have the same chromosome number $2n = 16$, with base chromosome number of 8 (Ladizinsky and Adler, 1976; Singh and Jauhar, 2005). Also, studies have reported nuclear DNA contents of annual *Cicer* sp. (Table 1) (Arumuganathan and Earle, 1991; Galasso et al., 1996; Ohri and Pal, 1991; Ruperao et al., 2014). Despite the constant chromosome number, the amount of DNA in the species has been reported to range from 1.53 to 3.57 pg per 2C nucleus (Arumuganathan and Earle, 1991; Galasso et al., 1996; Ohri and Pal, 1991; Ruperao et al., 2014). Although this could be due to intrachromosomal DNA variation, a concomitant chromosome size variation was not observed by the previous study (Ladizinsky and Adler, 1976). This leads to speculation that there may be errors in reporting genome size variation in the genus *Cicer*. Giving credence to this hypothesis is that two different 2C genome sizes have been reported in *C. arietinum*. Ohri and Pal (1991) and Galasso et al. (1996) obtained 3.57 and 3.29 pg for 2C genome sizes of *C. arietinum*, respectively, whereas Arumuganathan and Earle (1991) and Ruperao et al. (2014) reported 1.53 and 1.77 pg, respectively. Given these discrepancies in the literature, it would be prudent to confirm which, if any, of these estimates is more accurate. Such estimates are critical when correlating genome size variation with phenotypic variation.

The variation in genome size influences numerous cellular parameters such as chromosome size, nuclear volume, and cellular volume (Bennett, 1987; Grant, 1987), which has been called nucleotide effects (Bennett,

1972). The nucleotide effects can cause variation in phenotypic traits such as seed mass, and many previous studies have investigated the nucleotide effects on seed size and mass (Beaulieu et al., 2007; Bennett, 1987; Benor et al., 2010; Biradar et al., 1994; Chung et al., 1998; Knight and Ackerly, 2002). Most of the previous studies have indicated a positive relationship between genome size and seed mass within species and across species from the same genus and family (Benor et al., 2010; Biradar et al., 1994; Chung et al., 1998; Knight and Ackerly, 2002). However, in some cases, triangular (Bennett, 1987) and quadratic types of relationship between genome size and seed size (Beaulieu et al., 2007) were revealed in 1222 angiosperm species including, *Allium* and *Vicia*, with species with large genomes tending to have large seed mass, whereas species with small genome sizes having either large or small seed mass. However, the correlation between genome size and seed mass may be disrupted by years of selective breeding/artificial selection. According to previous studies (Doebley et al., 2006; Zhao et al., 2015), during the domestication of crops, the nucleotide diversity associated with the selected trait in crops is expected to be lower than that of the wild crops, due to strong selective cultivars over many years of generations. A similar result has been observed in cultivated *C. arietinum*. Seeds of *C. arietinum* can be phenotypically classified into two seed types: *desi* (small angular and dark colored) and *kabuli* (large owl-shaped cream colored) (Choudhary et al., 2012; van der Maesen, 1972). The *desi* types are mostly distributed in the Indian subcontinent, whereas the *kabuli* types are grown in the Mediterranean regions. Despite geographic divergence and phenotype differences, no significant difference in DNA content between the two seed types of *C. arietinum* were observed by Ohri and Pal (1991). However, until the issues of genome size estimates in *C. arietinum* are resolved, the reported nonassociation between genome size and seed type can be questioned. It is also important to examine the relationship between genome size and seed mass across wild *Cicer* sp. for developing an understanding of nucleotide effects with regard to artificial and natural selection in *Cicer* sp.

In this study, we used flow cytometry to analyze nuclear DNA content of 13 *Cicer* sp. to provide valuable new genomic information for both genomic analysis and plant breeding strategies for cultivated plant improvement. Seed type and mass of *Cicer* sp. were investigated, and then the relationship between genome size and seed type/mass was examined within groups of cultivated *C. arietinum* and wild *Cicer* sp.

Materials and Methods

Plant material. Seeds of cultivated *C. arietinum* and 12 wild *Cicer* sp. (Table 2) were obtained from the National Plant Germplasm Resource Laboratory/USDA, ARS, National Genetic Resource Program (Beltsville, MD) (Table 2). The genotypes

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Table 1. Chromosome number, plant type, and genome size of *Cicer* sp. obtained in previous studies. The superscript lower case alphabet indicates the references.

Species	Chromosome number	Plant type ^z	2C nuclear DNA content (pg)
<i>Cicer arietinum</i>	2n = 2x = 16 ^z	Annual	3.57 ^x , 3.29 ^w , 1.53 ^v , 1.77 ^u
<i>Cicer bijugum</i>	2n = 2x = 16 ^z	Annual	2.54 ^x
<i>Cicer canariense</i>	2n = 2x = 16 ^y	Perennial	—
<i>Cicer chorassanicum</i>	2n = 2x = 16 ^y	Annual	—
<i>Cicer echinospermum</i>	2n = 2x = 16 ^z	Annual	2.7 ^x and 2.61 ^w
<i>Cicer judaicum</i>	2n = 2x = 16 ^z	Annual	1.83 ^x
<i>Cicer multijugum</i>	—	Perennial	—
<i>Cicer nuristanicum</i>	—	Perennial	—
<i>Cicer pinnatifidum</i>	2n = 2x = 16 ^z	Annual	2.56 ^x
<i>Cicer pugins</i>	2n = 2x = 16 ^y	Perennial	—
<i>Cicer reticulatum</i>	2n = 2x = 16 ^z	Annual	2.65 ^{x,w}
<i>Cicer songaricum</i>	2n = 2x = 16 ^z	Perennial	—
<i>Cicer yamashitae</i>	2n = 2x = 16 ^y	Annual	—

^zLadizinsky and Adler (1976).

^ySingh and Jauhar (2005).

^xOhri and Pal (1991).

^wGalasso et al. (1996).

^vArunmuganthan and Earle (1991).

^uRuperao et al. (2014).

— = no information available.

included 15 accessions for cultivated *C. arietinum* including PI 193782, PI 193767, PI 359817, PI 360659, PI 451607, PI 360662, PI 360663, PI 374079, PI 374090, PI 420908, PI 426190, PI 426551, PI 458870, W6 3125, and W6 32898 and 12 wild species consisting of 1 accession for each *C. bijugum* (PI 458552), *Cicer canariense* (PI 557453), *Cicer chorassanicum* (PI 458553), *C. echinospermum* (PI 599041), *C. judaicum* (PI 458559), *Cicer multijugum* (PI 599085), *Cicer nuristanicum* (PI 604497), *Cicer pugins* (W6 14191), and *Cicer yamashitae* (PI 540657) and 2 accessions for each *C. pinnatifidum* (PI 458555 and PI 458556), *C. reticulatum* (PI 599052 and 36, PI 599050), *Cicer songaricum* (PI 599053 and PI 599074). The seeds were grown in potting soil under 24 h light at 26 °C. About 2–12 plants per *Cicer* accession were used for analysis of 2C genome size. Due to low germination rate, only

Table 2. Species accession, 2C genome size (mean ± SD), seed mass per 10 seeds (mean ± SD), seed type, and geographic origin of the *Cicer* accessions used in this study. Two different seed sizes were observed in desi (small and big) and kabuli (medium and large).

Species accession ^z	2C genome size (pg)	Seed mass (g) per 10 seeds	Seed type	Origin
<i>Cicer arietinum</i>				
PI 193782	1.69 ± 0.049	1.28 ± 0.060	desi (small)	Ethiopia
PI 193767	1.70 ± 0.056	2.3 ± 0.220	desi (big)	Ethiopia
PI 359817	1.67 ± 0.017	1.38 ± 0.066	desi (small)	India
PI 360659	1.72 ± 0.042	2.49 ± 0.560	desi (big)	Israel
PI 451607	1.69 ± 0.031	1.40 ± 0.140	desi (small)	Iran
PI 360662	1.66 ± 0.037	1.50 ± 0.080	desi (small)	Italy
PI 360663	1.66 ± 0.040	1.26 ± 0.060	desi (small)	Mexico
PI 374079	1.74 ± 0.065	2.73 ± 0.070	desi (big)	Bulgaria
PI 374090	1.68 ± 0.034	3.42 ± 0.220	kabuli (medium)	Morocco
PI 420908	1.71 ± 0.027	3.34 ± 0.140	kabuli (medium)	Jordan
PI 426190	1.76 ± 0.047	1.49 ± 0.100	desi (small)	Afghanistan
PI 426551	1.68 ± 0.045	1.25 ± 0.090	desi (small)	Pakistan
PI 458870	1.69 ± 0.029	3.94 ± 0.450	kabuli (medium)	California, United States
W6 3125	1.66 ± 0.051	1.28 ± 0.003	desi (small)	Nepal
W6 32898	1.71 ± 0.032	4.15 ± 0.200	kabuli (large)	Canada
<i>Cicer bijugum</i>				
PI 458552	1.31 ± 0.050	0.82 ± 0.040	—	Tukey
<i>Cicer canariense</i>				
PI 557453	1.66 ± —	0.37 ± 0.020	—	Spain, Canary Islands
<i>Cicer chorassanicum</i>				
PI 458553	1.35 ± 0.070	0.15 ± 0.014	—	Afghanistan
<i>Cicer echinospermum</i>				
PI 599041	1.63 ± 0.057	0.83 ± 0.030	—	Diyarbakir, Turkey
<i>Cicer judaicum</i>				
PI 458559	1.00 ± 0.096	0.21 ± 0.007	—	Israel
<i>Cicer multijugum</i>				
PI 599085	1.64 ± 0.068	0.45 ± 0.027	—	Uzbekistan
<i>Cicer nuristanicum</i>				
PI 604497	1.63 ± 0.057	0.60 ± 0.075	—	Pakistan
<i>Cicer pinnatifidum</i>				
PI 458555	1.40 ± 0.027	0.28 ± 0.003	—	Turkey
PI 458556	1.35 ± 0.056	0.22 ± 0.010	—	Turkey
<i>Cicer pugins</i>				
W6 14191	1.58 ± 0.029	0.88 ± 0.024	—	Afghanistan
<i>Cicer reticulatum</i>				
PI 599052	1.66 ± 0.030	1.04 ± 0.029	—	Siirt, Turkey
PI 599050	1.64 ± 0.015	0.75 ± 0.057	—	Siirt, Turkey
<i>Cicer songaricum</i>				
PI 599053	1.56 ± 0.014	0.79 ± 0.017	—	Uzbekistan
PI 599074	1.62 ± 0.027	0.60 ± 0.020	—	Tajikistan
<i>Cicer yamashitae</i>				
PI 540657	1.13 ± 0.060	0.29 ± 0.001	—	Afghanistan
LSD	0.0587	0.2567		
P value	<0.0001	<0.0001		

LSD = least significant difference.

^zAccession obtained from the NPGRL/USDA, ARS, National Genetic Resource Program.

— = no information available.

Fig. 1. Flow histograms of *Cicer* sp. somatic nuclei stained with PI. The bar represents the nuclei used to calculate the mean fluorescence of each peak. (A) G1 somatic nuclei of *Cicer arietinum*. (B) G1 somatic nuclei of *Cicer bijugum*. (C) G1 somatic nuclei of *Cicer judaicum*.

one plant for *C. canariense* (PI 557453) was used for analysis of 2C genome size.

Analysis of genome size. To determine 2C genome size, flow cytometric analysis was performed using the protocol described by Kim et al. (2010). Fresh stem tissues (≈ 5 cm² of each) from *Cicer* sp. and an internal standard were co chopped, and placed in 10 mL of extraction buffer and 200 μ L of 25% Triton X. The extraction buffer consisted of 13% hexylene glycol, 10 mM Tris-HCl (pH 8.0), and 10 mM MgCl₂. The use of the internal standard provides a relative measure of the 2C genome size of the sample. Maize was used as an internal standard because the 2C genome size of maize does not overlap with the targeted genome size

of *Cicer* sp. The 2C genome size of maize (VT3 subpopulations) was calibrated at 5.15 pg using sorghum (Pioneer hybrid 84G62) with 1.74 pg/2C nuclei (Rayburn et al., 2009). For each sample, the tissue was homogenized using a tissue grinder for 10 s at 4500 g_n, and the samples were filtered through a 50- μ m filter (Partec, GmbH, Münster, Germany) into a test tube. After centrifuging for 15 min at 11,000 g_n at 4 °C, the supernatant was removed, and the nuclei were suspended in 300 μ L of PI stain (3% w/v polyethylene glycol (PEG) 6000, 5 μ g·mL⁻¹ of PI, 180 units/mL RNase, 0.1% Triton X-100 in 4 mM citrate buffer). The solution was transferred to a 1.5-mL tube and incubated in a water bath for 20 min at 37 °C. PI salt stain (3% w/v PEG

6000, 5 μ g·mL⁻¹ of PI, and 0.1% Triton X-100 in 400 mM NaCl) was added, and the tube was cooled to 4 °C for at least 1 h.

A BD LSR II flow cytometer (BD Biosciences, San Jose, CA) was used to analyze the stained nuclei. The excitation wavelength was set at 488 nm. The emission filter was a 695/40 nm-filter. At least 30,000 nuclei per sample were screened. The nuclei were gated on the basis of fluorescence integral vs. pulse width to exclude the doublets. The total 2C genome size reported in this study is the amount of nuclear DNA in somatic G1 nuclei of each plant.

Seed analysis. Seed photographs were taken using a color camera (DP22; Olympus, Shinjuku, Tokyo, Japan) through a zoom stereo microscope (S2-CTV; Olympus). The seed mass of three

Fig. 2. The seed pictures of *Cicer* sp. Two different seed types are shown in *Cicer arietinum*: *desi* (PI 360659 and PI 360663) and *kabuli* (PI 458870). The size of bar is 1 cm.

replicates of 10 seeds per *Cicer* accession were measured.

Statistical analysis. All statistical analyses were carried out using SAS 9.3 (SAS institute Inc., Cary, NY). General linear model analysis was conducted and Fisher's protected least significant difference test was run to determine the significant difference ($\alpha = 0.05$) among all *Cicer* accessions in 2C genome size and seed mass. The relationship between means of genome size and seed mass plotted on raw-scaled axes (Niklas, 1994), which was carried out separately on accessions of the cultivated species (*C. arietinum*) and on accessions of wild *Cicer* sp. Bivariate trait relationships were

analyzed by fitting a linear regression trend line using the least squares fit method (Herkimer, 1986). The linear relationship is expressed as $Y = \alpha + \beta X$, where Y and X stand for means of variables of seed mass and genome size, respectively, and α and β represent the intercept on the Y axis and the regression coefficient, respectively. F statistics were used for testing the null hypothesis that the regression coefficients are all equal to zero ($\alpha = 0.05$).

Results and Discussion

A significant difference in the average of 2C genome size was observed among *Cicer* sp.

ranging from 1.00 (*C. judaicum*) to 1.76 pg (*C. arietinum*) ($P < 0.0001$) (Table 1; Fig. 1). Compared with perennial *Cicer* sp., a larger range of 2C genome size was observed among the annual *Cicer* sp. (Tables 1 and 2). Similar results have been observed by Ohri and Pal (1991) who reported a significant difference in 2C genome size among six annual *Cicer* sp. However, there are some discrepancies between the previous data and ours; in previous 2C genome size estimations (Table 1) (Galasso et al., 1996; Ohri and Pal, 1991), the results obtained were in most cases larger than observed in our study (Table 2). Some noticeable differences in the data on

Fig. 3. The relationship between means of seed mass and means of genome size across 12 wild *Cicer* sp. (seed mass = 0.8157, genome size = 0.6533, $R^2 = 0.3528$, $P = 0.0196$).

2C genome size were also observed in *C. arietinum*, *C. bijugum*, *C. echinospermum*, *C. judaicum*, *C. pinnatifidum*, and *C. reticulatum*. In these cases, previous estimations differed from ours by up to 80%. Unlike the previous estimation, done by Ohri and Pal (1991) and Galasso et al. (1996), the 2C genome size of *C. arietinum* observed by Arumuganathan and Earle (1991) and Ruperao et al. (2014) is similar to our results (Table 2). In addition, the draft whole genome sequence of *C. arietinum* has been reported to be ≈ 738 Mb, which converts to a 2C genome size of ≈ 1.5 pg (Varshney et al., 2013). Given these genome size results, a reassessment of the genome size for wild species was addressed.

The 2C genome size for *C. arietinum*, *C. bijugum*, *C. echinospermum*, *C. judaicum*, *C. pinnatifidum*, and *C. reticulatum* were observed around 1.7, 1.31, 1.63, 1.00, 1.38, and 1.65 pg, respectively (Table 2). In addition, the 2C genome sizes for other seven wild *Cicer* sp. including five perennial (*C. canariense*, *C. multijugum*, *C. nuristanicum*, *C. pugens*, and *C. songaricum*) and two annual species (*C. echinospermum* and *C. yamashitae*) were around 1.66, 1.64, 1.63, 1.58, 1.6, 1.63, and 1.13 pg, respectively (Table 2). Both in cultivated and wild *Cicer* sp., the genome size estimates were in agreement with lower genome sizes of *C. arietinum*. In addition, the chromosome size data of Ohri and Pal (1991) is more consistent with the genome sizes obtained in this study. Whether this is due to differences in technologies between flow cytometry and Feulgen DNA microdensitometry, internal standard species, technical issues, or other unknown factors could not be addressed. Given the three previous studies agree with the lower genome size of *C. arietinum* (Arumuganathan and Earle, 1991; Ruperao et al., 2014; Varshney et al., 2013), the association of seed characteristics with genome size was reevaluated.

Seed phenotypes such as size, color, shape, and seedcoating varied within *C. arietinum* and among *Cicer* sp. (Fig. 2). Within *C. arietinum*, the two distinctive seed types, such as *desi* and *kabuli*, were distributed widely over geographic regions (Table 2). Two different seed sizes were found in *desi*

types (Fig. 2). The range of seed mass for the bigger size of the *desi* type was 2.3–2.5 g, whereas the range of seed mass for the smaller size was 1.25–1.5 g/10 seeds (Table 2; Fig. 2). The seed size of the *kabuli* seed type also varied. According to Moreno and Cubero (1978) and Choudhary et al. (2012), the *kabuli* seeds used in this study can be classified into medium seeded (range 3.3–3.94 g/10 seeds) and large seeded (4.15 g/10 seeds) (Table 2). Seed phenotypes also varied among the wild species. Seed of wild perennial species are mostly round, triangular, shaped, and darker colored, but various seed sizes were found; higher seed phenotypic variations occurred within wild annual *Cicer* sp. (Fig. 2). Seed of *C. reticulatum*, which is well known as the wild progenitor of *C. arietinum* (Ahmad et al., 1987), has a mix of characteristics of *desi* (angular seed shape) and *kabuli* (cream color). *Cicer bijugum* has dark reddish colored hairy seedcoat, whereas *C. echinospermum* has a short yellowish colored hairy seedcoat (Fig. 3). Overall, the seed sizes of wild species were smaller than the two seed types of *C. arietinum* (Table 2), which reflects lower seed weights in wild species (Table 2).

The relationship between 2C genome size and seed mass was investigated within groups of the cultivated *Cicer* sp. (*C. arietinum*) and 12 wild *Cicer* sp. Because the cultivated species have been subjected to many years of artificial selection by plant breeders, the relationship between 2C genome size and seed mass of cultivated and wild *Cicer* sp. was considered separately. We found no correlation between 2C genome size and seed type within 13 lines of *C. arietinum* including the two seed types, *desi* and *kabuli* (seed mass = 11.436, genome size = 17.187, $R^2 = 0.107$, $P = 0.233$). The range of the 2C genome size within *C. arietinum* was between 1.66 and 1.76 pg (Table 2). The seed types having both highest and lowest genome sizes were *desi* (Table 2). This result is also supported by Ohri and Pal (1991) who reported that no significant difference in DNA content was observed between *desi* and *kabuli* cultivars of *C. arietinum*, despite using the inflated genome size. However, a significant linear

relationship was found between 2C genome size and seed mass among 12 wild *Cicer* sp. (seed mass = 0.8157, genome size = 0.6533, $R^2 = 0.3528$, $P = 0.0196$) (Fig. 3). For example, *C. judaicum* had the lowest genome size and also had lower seed mass. This strong positive relationship between genome size and seed mass in plants has also been observed in many previous studies (Bennett, 1972; Grime et al., 1997; Knight and Ackerly, 2002; Thompson, 1990). As genome size increases, the cell size within seed organs may get bigger, resulting in increase in seed mass (Beaulieu et al., 2008). Artificial selection appears to have disrupted the natural selection between genome size and seed mass in cultivated *C. arietinum*.

In conclusion, the 2C genome size estimations in this study confirm the previously reported findings, showing that the 1.5 (Arumuganathan and Earle, 1991) and 1.7 pg (Ruperao et al., 2014) are the more accurate 2C genome sizes of *C. arietinum*, and that other estimated genome sizes of 3.29 and 3.57 pg (Galasso et al., 1996; Ohri and Pal 1991) were likely an overestimation. In addition, the genome sizes for wild *Cicer* sp. estimated in this study were lower than the previous 2C genome sizes estimated in Ohri and Pal (1991) and Galasso et al. (1996). Significant differences in genome size were observed among 13 *Cicer* sp. The 2C genome size ranged from 1.00 pg in wild species, *C. judaicum*, to 1.76 pg in cultivated species, *C. arietinum*. Compared with perennial *Cicer* sp., the 2C genome size more varied within annual *Cicer* sp.. Nucleotype effects appear to result in phenotypic variation across wild *Cicer* sp., but not across cultivated *C. arietinum*. No correlation between genome size and seed size was observed within *C. arietinum*, but a strong positive relationship between genome size and seed size was observed among 12 wild *Cicer* sp. By understanding the role of nucleotype and seed size, the potential exists to develop novel breeding schemes for *C. arietinum* improvement.

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