Cytogenetics of *Cicer*

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Abstract

Chickpea (*Cicer arietinum*) is among the most widely grown grain legumes, with the major growing area concentrated in the Indian subcontinent. The species is diploid ($2n = 2x = 16$) and is the only domesticated species in a genus, which includes over 40 annual and perennial species. The progenitor of the cultivated form is the annual species *C. reticulatum,* but both annual and perennial relatives have been considered as donors of useful genetic variation. Recent advances in genomic analysis have expanded the results of earlier cytogenetic research in the species, which established base information with respect to the karyotype (chromosome number, length, and morphology; and some limited descriptions based on banding) and an estimate of nuclear genome size. Chromosome behavior at meiosis has been characterized in a few *Cicer* species and some wide hybrids. To date, only a small number of DNA sequences have been chromosomally localized using in situ hybridization. No detailed cytogenetic map has been elaborated, and the level of knowledge regarding the long-range molecular chromosomal organization of the genome is rudimentary. A recently developed method for sorting chickpea chromosome using flow cytometry now offers a more effective means of exploring the genome.

4.1 Introduction

The genus *Cicer*, which belongs to the Fabaceae family, is the sole genus in the tribe Cicereae. Considerations of life cycle, morphology, and geographical distribution have allowed the 43 *Cicer* species to be classified into the four groups Monocicer, Chamaecicer, Polycicer, and Acanthocicer. Eight of the nine annual species (the

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exception is *C. chorassanicum*), which include the cultivated form *C. arietinum*, belong to the section Monocicer (van der Maesen 1987). Chickpea is the only cultivated *Cicer* species and, in terms of production and consumption, is among the most important grain legumes. It is important as a source of protein in the vegetarian diet, particularly in the Indian subcontinent, where the bulk of production (72% in quantity terms in 2014, according to FAOSTAT (http://faostat.fao.org/)); a further 6% of the cropping area is in western Asia. The crop is adapted to low rainfall conditions, but drought has been identified as one of the most important constraints to productivity.

The origin of chickpea has been traced to Turkey, in an area harboring most of the wild *Cicer* species, including the annual *C. reticulatum*, identified as the likely progenitor of the cultivated type (Ladizinski and Adler 1976b). Two distinct market classes are produced: The seed of kabuli types is large, non-pigmented, and smooth, while desi-type seed is rough, angular-shaped, and dark-colored. Kabuli plants lack anthocyanin pigmentation on their stem, while desi plants form pigmented stems and its flowers are pink (Pundir et al. 1985). Despite the importance of cultivated chickpea, little effort has been made to date to explore its genome at the chromosomal level, and even less with respect to that of its close relatives. Karyotypic descriptions and an estimate of the nuclear DNA amount date back at least 20 years (Ohri and Pal 1991; Ocampo et al. 1992; Galasso and Pignone 1992).

While most *Cicer* species are perennial, the cultivated form is an annual plant. The species has been described as preadapted to domestication (Ladizinsky 1979). The domestication process itself required the loss of pod shatter, a change in growth habit from a prostrate stem to a semierect to erect stem, the loss of vernalization requirement, a reduction in seed dormancy, and changes to seed size, shape, and color (Abbo et al. 2014; Gupta and Bahl 1983). The negligible economic importance of the perennial *Cicer* spp. has left these at best only superficially described, with the consequence that almost nothing is known regarding their evolution and phylogenetic relationships. At the same time, a number of both annual and perennial relatives have been exploited as donors of useful genetic variation with a view to chickpea improvement (Haware and McDonald 1992; Collard et al. 2001; Sharma et al. 2006; Singh et al. 2005).

4.2 Ploidy and Chromosome Number

The *Cicer* species are uniformly diploid, all showing a somatic chromosome number of 16 (Ladizinski and Adler 1976a; Ocampo et al. 1992). While there is no available evidence for any recent polyploidization event(s), gene copy number variation in the Fabaceae has suggested that such events have influenced the form of the chickpea genome, in particular, the legume-wide whole genome duplication predicted to have occurred about 58 million years ago (Jain et al. 2013). The rate of synonymous substitution per site per year has been estimated to be 6.05×10^{-9} (Jain et al. 2013), a frequency some 12% more rapid than is the case in *Medicago* (Young et al. 2011). The absence of any recent whole genome duplication affecting the genera *Medicago*, *Cicer*, and *Lotus* (Young et al. 2011; Sato et al. 2008; Jain et al. 2013) implies that speciation within the Fabaceae has not been driven by abrupt changes in chromosome number, but rather by chromosomal rearrangements and/or lineage-specific gene gains/losses. The diploid status of the *Cicer* species is mirrored in the genera *Lens*, *Pisum*, and *Vicia*, which belong to the related tribe Fabeae: each have a similar somatic chromosome number to that of the *Cicer* spp: in both *Lens* and *Pisum*, this is 14 (Mishra et al. 2007), while in *Vicia*, diploid species have basic chromosome numbers $x = 5$, 6 and 7 (Kaur and Singhal 2010). Species belonging to a fourth-related genus (*Lathyrus*) harbor many more chromosomes: Two *Lathyrus* species are tetraploid ($2n = 28$) and one is hexaploid ($2n = 48$) (Campbell and Clayton 1997).

4.3 Chromosome Morphology

The chickpea chromosomes are small (Fig. 4.1): The mean length of the mitotic metaphase chromosomes is around 2.2 μm (Ahmad 2000), which translates to a nucleotide content of slightly over 100 Mbp, equivalent to an eighth of the size of the average wheat chromosome (Šafář et al. 2010), but twice that of those of banana (Doležel et al. 1994). Although the somatic chromosome number of the annual *Cicer* species is invariant, there is plenty of karyological variation, and the same probably holds for the perennial species. Ahmad (2000), in a study of all nine annual *Cicer* species, recorded differences with respect to both chromosome length and the position of primary and secondary constrictions; these differences were significant enough to rule out proposing a unified karyotype across the annual *Cicer* species. Such variation supports the notion that structural alterations to the chromosomes likely have driven evolution and speciation within *Cicer*. There is even some evidence for intraspecific karyotypic variation (Ohri and Pal 1991; Ocampo et al. 1992; Tayyar et al. 1994; Ahmad 2000; Ahmad and Hymowitz 1993; Kordi et al. 2006), since these various authors are in disagreement regarding chromosome length, arm ratio, and the position of the secondary constriction. According to Ahmad (2000), however, these discrepancies may well be artifacts arising from inconsistencies in the cytological protocols.

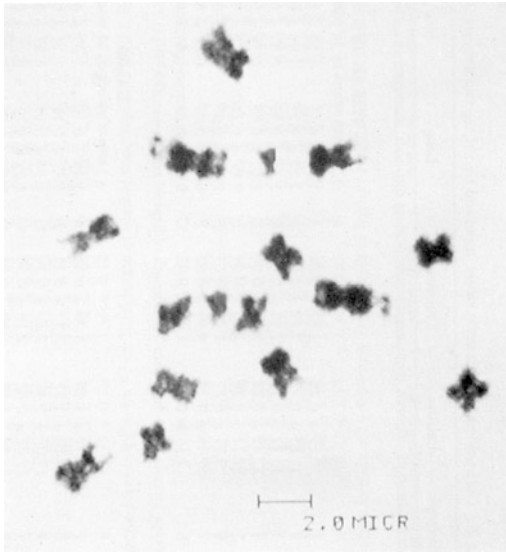

Fig. 4.1 A metaphase plate of cultivated chickpea. Copied from Venora et al. (1995) with the permission of the publisher (Karyotype of kabuli-type chickpea (*Cicer arietinum* L.) by image analysis system. Venora G, Ocampo B, Singh KB, Saccardo F. Caryologia, copyright ©University of Florence, reprinted by permission of Taylor & Francis Ltd., www.tandfonline.com on behalf of University of Florence

Chromosomes associated with the nucleolus organizing region (NOR) are readily recognized as they form a secondary constriction. Typically, only one chromosome pair in *Cicer* species shows this structure (Ohri and Pal 1991; Tayyar et al. 1994; Kordi et al. 2006). The sole exception is *C. reticulatum,* which harbors two pairs of satellited chromosomes (Ohri and Pal 1991; Ocampo et al. 1992). The silver stain assay was used by Galasso et al. (1996) to demonstrate that both NOR loci are active, albeit not equally. The conclusion was that during the evolution of *C. echinospermum* and *C. arietinum* from *C. reticulatum,* one of the two NOR loci was lost. However, Ahmad (2000) was unable to confirm the presence of two satellited chromosome pairs in *C. reticulatum* and suggested the possibility that two cytotypes of *C. reticulatum* exist, one with a single NOR locus and the other with two. Cultivated types bearing two NOR chromosome pairs have been reported in the early literature (Iyengar 1939; Kutarekar and Wanjari 1983) but have not been confirmed in more recent work. Some *C. arietinum* accessions reportedly display a tandemly arranged pair of satellites on the largest chromosome of the complement, but these only appear during late prophase/early metaphase (Meenakshi and Subramaniam 1960; Ahmad 1989, 2000; Tayyar et al. 1994; Kordi et al. 2006). The various explanations for this phenomenon have included staining artifacts (Ohri and Pal 1991; Ocampo et al. 1992; Galasso and Pignone 1992), NOR movement (Schubert 1984; Schubert and Wobus 1985), and evolutionary rearrangements (Ladizinski and Adler 1976b; Galasso et al. 1996; Kordi et al. 2006), but none of these are totally satisfactory.

The location of the secondary constriction varies among the *Cicer* species: In the annuals *C. arietinum, C. reticulatum,* and *C. echinospermum*, it is present on the longest chromosome pair, while in the others, it is associated with a medium- or a small-sized chromosome (Ahmad 2000).

With the exception of secondary constriction, chickpea chromosomes do not show marked features, which could ease their classification. Chromosome length at mitotic metaphase lies in the range from 1.32 to 3.69 μm (Ahmad 2000); three of the chromosomes are submetacentric and the others metacentric (Fig. 4.2). There is a suggestion of differences in relative chromosome length between the chromosomes of the kabuli and desi types; in the former, three of the chromosomes appear longer than their equivalents in the latter type, while the other five appear to be longer in desi types, but these differences are small, ranging from 0.2 to 0.8% of the overall relative chromosome length (Ruperao et al. 2014) (Table 4.1). Although this variability in chromosome length is consistent with the observations of Kordi et al. (2006), it is less substantial than was claimed by Ohri and Pal (1991). Only two of the eight *C. arietinum* chromosomes can be unambiguously identified based on their morphology: These are the longest submetacentric chromosome which bears the NOR, and the shortest metacentric one (Kordi et al. 2006). Except for the longest and shortest chromosomes, which are always classified as being, respectively, submetacentric and metacentric, according to Kordi et al. (2006), at least one of the six remaining chromosomes departs from the mean length and/or arm ratio assigned to the reference accession by Ahmad (2000). Moreover, it is evident that the karyotype of cultivated chickpea is more distinctive and the differences in the length of individual chromosomes are bigger as compared to other annual species (Ahmad 2000).

Karyotype symmetry, as defined by Stebbins (1971), has some value as a descriptive parameter. The concept defines four levels of asymmetry in the placement of the centromere and three in the length of individual chromosomes. According to this system, there exist two types of asymmetry among the annual *Cicer* species (Ahmad 2000): One group clusters *C. arietinum*, *C. reticulatum*, and *C. echinospermum* and supports conclusions based on crossability, phylogenetic and genotypic (molecular marker-based) diversity analyses (Buhariwalla et al. 2005; Iruela et al. 2002; Sudupak et al. 2004).

Two chromosome naming systems have been used in *Cicer*, one based on numbers from 1 (longest chromosome) to 8 (shortest) (Ocampo et al. 1992), and the other on letters (*A*–*H*), where $A = 1$, $B = 2$, etc. (Galasso et al. 1996; Staginnus et al. 1999; Vláčilová et al. 2002; Zatloukalová et al. 2011). Both systems were in use until the first linkage maps were assembled, after which they were replaced by linkage group (LG) numbers. A system based on pseudomolecules has recently been proposed by Ruperao et al. (2014) (Table 4.1).

Little attempt has been made to cytogenetically characterize the perennial *Cicer* species. In 1972, van der Maesen estimated their chromosome number to be either $2n = 14$ or $2n = 16$ (van der Maesen 1972). The first description of the karyotype of a perennial *Cicer* species involved *C. anatolicum* (Ahmad 1989),

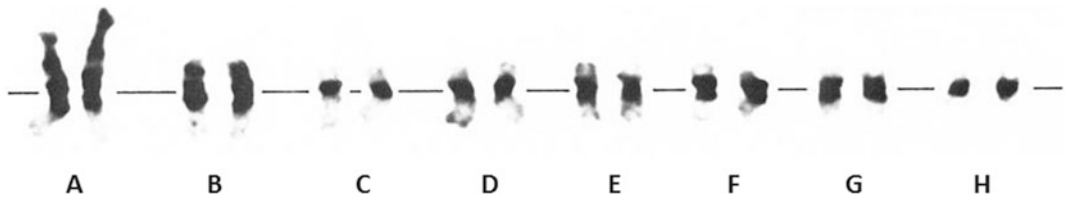

Fig. 4.2 C-banding karyotype of chickpea. Copied from Galasso and Pignone (1992) with the permission of the publisher (Characterization of chickpea chromosomes by banding techniques. Galasso I and Pignone D. Genetic Resources and Crop Evolution, copyright ©Kluwer Academic Publishers. With permission of Springer)

Table 4.1 Chickpea desi and kabuli chromosome nomenclature, their assignment to linkage groups, and individual chromosome sizes as determined from cytological data. Pseudomolecule number (Ca) corresponds to the linkage group number (LG). (Adapted from Ruperao et al. 2014)

Cicer arietinum L.					
Chromosome	Pseudomolecule	Relative chromosome length (%)		Molecular chromosome size (Mbp)	
		Desi "4958"	Kabuli "Frontier"	Desi "4958"	Kabuli "Frontier"
A	Ca5	19	19.8	164.92	174.64
B	Ca3	15.8	16.7	137.14	147.29
C	Ca6	13.3	12.9	114.58	112.9
D	Ca7	12.6	11.8	109.37	104.01
E	Ca4	11.5	11.1	99.82	97.9
F	Ca2	10.7	10.5	92.88	92.61
G	Ca1	9.9	9.4	85.93	83.91
H	Ca8	7.2	7.8	62.5	68.8
Total		100	100	867.14	882.06

establishing $2n = 16$ as the chromosome number, as is the case for the annuals. Subsequent analysis showed that the karyotype of *C. songaricum* was even more similar to that of *C. arietinum*, *C. reticulatum*, and *C. echinospermum,* at least in central and distal parts of the chromosomes.

4.4 Nuclear Genome Size

Similarly to the shortage of systematic studies on karyotype within *Cicer*, there are only a few reports on estimation of nuclear DNA content. Despite the stable chromosome number in genus *Cicer*, there seem to be remarkable differences in nuclear DNA content among its species. The first estimation of nuclear DNA amount in chickpea was reported by Bennett and Smith (1976), who gave nuclear DNA amount of 1.9 pg/2C for *C. arietinium*. In a more recent study, Ruperao et al. (2014) verified DNA amounts in chickpea using flow cytometry and estimated 2C DNA amounts of kabuli and desi types to be 1.80 and 1.77 pg, respectively. The differences in 2C amounts between four accessions of desi type were negligible. Using these values, mean nuclear 1C genome sizes of kabuli and desi types were determined as 882 and 866 Mbp, respectively. In the largest study performed so far, Ohri and Pal (1991) determined DNA content in six annual *Cicer* species and five accessions of cultivated chickpea. Surprisingly, C-values of *C. arietinum* were much higher than those estimated by Bennett and Smith (1976) and Ruperao et al. (2014) (Table 4.2). Cultivated chickpea had the highest DNA amounts (2C = 3.3–3.57 pg) of all analyzed accessions. Estimates of 2C DNA content in all analyzed species ranged from 1.83 pg in *C. judaicum* to 3.57 pg in one of the cultivated chickpea accessions. DNA amount of perennial *C. songaricum* (2C = 2.72 pg) was comparable to that of *C. reticulatum* (2C = 2.66 pg) and C. *echinospermum* (2C = 2.6 pg). Some of the C value estimates were confirmed later by Galasso et al. (1996). It should be noted that both groups estimated DNA amounts using Feulgen microdensitometry and used *Vicia faba* and *Alium cepa*, respectively, as reference standards.

Clearly, there seem to be large inconsistencies in the estimates of nuclear DNA amount in *Cicer*. The reason for this is not clear, and a caution is warranted when using published data. For example, the karyotype of cultivated chickpea is similar to its wild progenitor, *C. reticulatum* (Ahmad et al. 1992; Iruela et al. 2002). Yet, the published data on 2C amounts in both species differ significantly (Table 4.2). It appears unlikely that a large change in DNA amount would occur during the process of domestication and cultivation of *C. arietinum* without marked

Table 4.2 Estimates of nuclear DNA amounts in species belonging to the genus *Cicer*

	Reference			
Annual species	Bennett and Smiths (1976)	Ohri and Pal (1991)	Galasso et al. (1996)	Ruperao et al. (2014)
C. arietinum "kabuli"	0.95	1.67	1.64	0.9
C. arietinum "desi"		1.65		0.89
C. bujungum K.H.Rech.		1.27		
C. cuneatum Hochst. Ex Rich.		1.25		
C. echinospermum P.H. Davis		1.35	1.3	
C. judaicum Boiss.		0.92		
C. pinnatifidum Jaub.& Sp.		1.28		
C. reticulatum Ladiz.		1.32	1.33	

	Reference
Perennial species	Ohri (1999)
C. songaricum	1.36

changes in chromosome length and morphology. With this limitation in mind, when the available DNA content estimates are compared to recent phylogenetic data, there seems to be a positive correlation between the difference in genome size and genetic distance (Ohri and Pal 1991; Buhariwalla et al. 2005).

4.5 Longitudinal Differentiation of Chromosomes

Similarities in chromosome size and morphology do not permit identification of individual chromosomes in chickpea. However, this can be achieved after a procedure called Giemsa C-banding, which stains preferentially heterochromatin regions. When applying this method to chickpea, Galasso and Pignone (1992) and Galasso et al. (1996) observed differences in the distribution of heterochromatin along individual chromosomes. C-banding pattern included strong bands around centromeres and occasional weak banding patterns in middle and distal parts of chromosome arms (Fig. 4.2). Except for *C. judaicum* and *C. pinnatifidum*, C-banding polymorphisms have provided the means to identify each individual chromosome pair (Tayyar et al. 1994). The use of fluorochromes differing in DNA base affinity (DAPI, Hoechst 33258, and Chromomycin A3) has revealed significant variability in heterochromatin content among the annual *Cicer* species. Tayyar et al. (1994) used these stains to arrive at an estimated heterochromatin content of 40% in most of the annual species, although the ratio rose to 60% in *C. cuneatum* and *C. bijungum.* The difference was thought to reflect a correlation between evolutionary advancement and heterochromatin reduction (Tayyar et al. 1994). However, attempts to group the species based on their heterochromatin content proved to be inconsistent with their grouping based on either crossability (Ladizinski and Adler 1976b) or alleles at isozyme (Kazan and Muehlbauer 1991) or seed storage protein (Ahmad and Slinkard 1992) loci.

The recent acquisition of the genome sequence of both the desi and kabuli types (Jain et al. 2013; Varshney et al. 2013; Parween et al. 2015) has facilitated the use of sequence-based markers to characterize the genetic diversity present both between and within wild and cultivated *Cicer* species. For example, Bajaj et al. (2015) exploited variation at >27,000 SNP loci distinguishes the cultivated type (both desi and

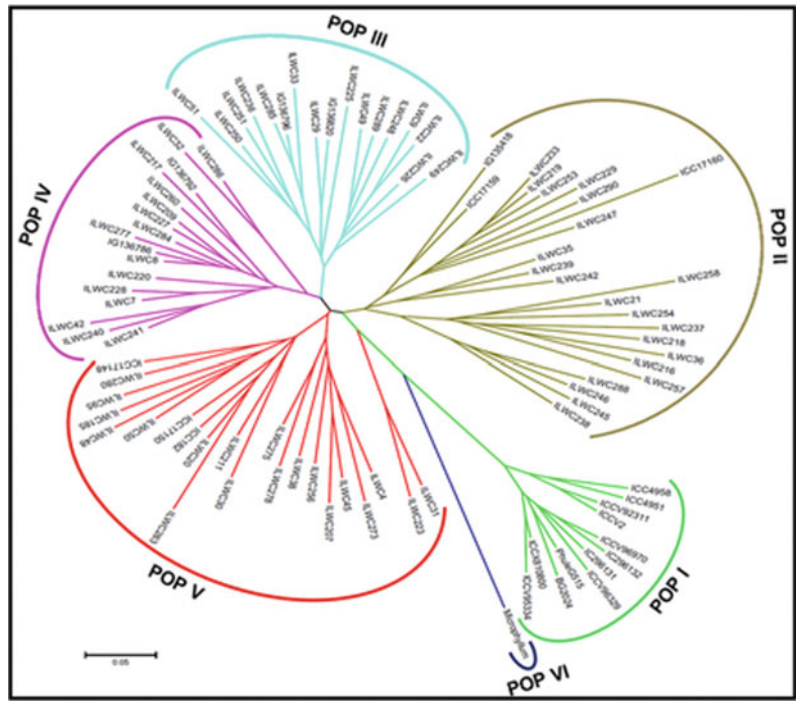

Fig. 4.3 Unrooted cladogram illustrating genetic relationships (Nei's genetic distance) among 93 wild and cultivated accessions belonging to seven *Cicer* species obtained using 27,862 genome-wide SNPs. The phylogenetic tree clearly differentiated 93 accessions into six diverse groups, which correspond to *Cicer* species and gene pools of origination. POP I consists of desi and kabuli accessions, POP II consists of the accessions of *C. reticulatum* and *C. echinospermun*, and other four distinct clades (POP III–VI) represent *C. judaicus*, *C. bijugum*, *C. pinnatifidun*, and *C. microphyllum* species. Genome-wide SNP-based molecular diversity, phylogeny, and population genetic structure among 93 wild and cultivated *Cic*er accessions by Bajaj et al. (2015), used under CC BY 4.0/excised from the original

kabuli) from accessions of *C. reticulatum* and *C. echinospermun*, and also from the more distant taxa *C. judaicus*, *C. bijugum*, *C. pinnatifidun*, and *C. microphyllum* (Fig. 4.3). Meanwhile, Kujur et al. (2015) showed that single nucleotide polymorphism (SNP)-based genotyping was able to divide a collection of cultivated germplasm into the two recognized major groups, kabuli and desi, and a detailed analysis of the SNP-based genetic diversity within these two groups has been presented by Upadhyaya et al. (2008), Roorkiwal et al. (2014), and Kujur et al. (2015).

4.6 Meiosis

Since the chromatin in a meiotic chromosome is less condensed than in a mitotic one, the former is more informative with respect to chromosome morphology and structure. As yet, however, meiotic chromosomes in the genus *Cicer* have not been systematically studied. Although Kabir and Singh (1991) observed some abnormalities, in general meiosis in cultivated chickpea was regular with eight bivalents formed in metaphase I. The character of the bivalents was more open (rod) than closed (ring), and chiasma frequency per pollen mother cell (PMC) was variable among the nine analyzed Cicer species (Ahmad and Chen 2000, Fig. 4.4). An analysis of pachytene chromosomes provided by Ahmad and Hymowitz (1993) exposed the distribution of heterochromatin along the chromosomes and confirmed that only one chromosome pair in *C. arietinum* was associated with the nucleolus; the chromosome arm carrying the NOR was highly heterochromatic, just as is the case in soybean

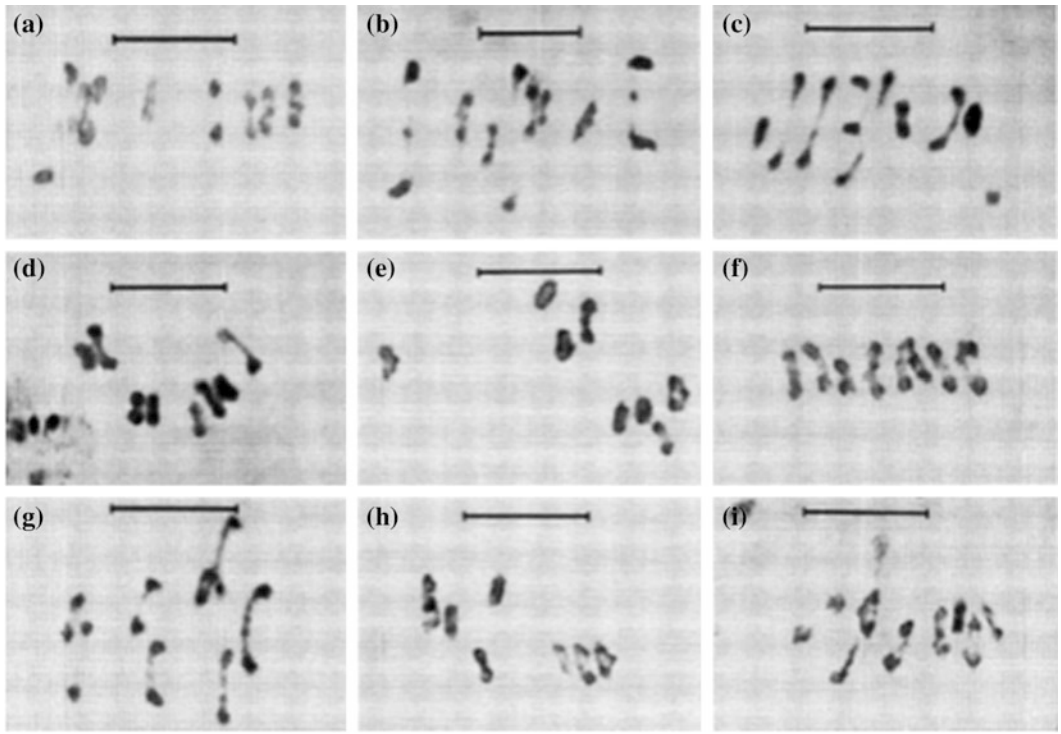

Fig. 4.4 Chromosome pairing at meiotic metaphase I in annual *Cicer* species. **a** *C. arietinum*, **b** *C. reticulatum*, **c** *C. echinospermum*, **d** *C. pinnatifidum,* **e** *C. judaicum*, **f** *C. bijungum*, **g** *C. chorassanicum*, **h** *C. yamashitae*, **i** *C. cuneatum*. Bar: 10 μm. The image has been taken from Ahmad and Chen (2000), with the permission of the publisher

(Singh and Hymowitz 1988), pigeon pea (Reddy 1981), and maize (McClintock 1929). The study also indicated that in the pachytene chromosome, the distinction between heterochromatin and euchromatin was clearer than in either barley (Singh and Tsuchiya 1975) or rice (Kush et al. 1984).

4.7 Molecular Cytogenetics

The elaboration of the fluorescence in situ hybridization (FISH) technique to localize specific DNA sequences on a mitotic or meiotic chromosome has generated important insights into chromosome organization in many organisms, including *Cicer* spp. The bulk of these experiments in *Cicer* has focused on the cultivated form, leaving the level of understanding of the chromosome organization in other *Cicer* species at best only limited. The ribosomal RNA genes were the first sequences to be localized in this way (Abbo et al. 1994; Staginnus et al. 1999). While only one chromosome pair carries a visible satellite, two sites hybridize with a 45S rDNA sequence, which was interesting in light of the presence of two satellited chromosome pairs in *C. reticulatum* (Ohri and Pal 1991; Abbo et al. 1994). Two sites harboring 5S rRNA sequences have been identified, one of which lies on the same chromosome as one of the 45S rDNA sites (chromosome B) (Vláčilová et al. 2002).

About 50% of the chickpea genome comprises repetitive DNA (Jain et al. 2013; Varshney et al. 2013). Some of the sequences within this fraction can be highly informative as cytogenetic markers, especially where their chromosomal distribution is non-random (Schwarzacher 2003; Jiang and Bikram 2006). FISH based on probe sequences detecting five distinct microsatellite motifs ($(A)_{16}$,

$(CA)_8$, $(TA)_9$, $(AAC)_5$, and $(GATA)_4$), which were selected based on results of previous study (Sharma et al. 1995), unfortunately failed to produce a chromosome-specific karyotype: The distribution and intensity of the signal varied from repeat motif to repeat motif, but all five were dispersed within each chromosome (Gortner et al. 1998). As anticipated, a telomeric sequence hybridized to each of the chromosome ends, but a weaker site in the pericentromeric region of chromosome A and a major cluster on the short arm of chromosome B were also evident (Zatloukalová et al. 2011; Staginnus et al. 1999). Nevertheless, the potential of repetitive DNA sequences has demonstrated in several studies. For example, the two tandemly organized chickpea-specific repeats (CaSat 1 and CaSat 2) isolated from a genomic library by Staginnus et al. (1999) were both informative: The former defined a large cluster of sites in the subtelomeric region of both chromosomes A and B, while the latter proved to be present at each of the eight centromeres. The retrotransposon-like sequences, CaRep 1, CaRep 2, and CaRep 3, derived from different parts of a Ty3/Gypsy-like element, are dispersed throughout the genome and produce a strong FISH signal concentrated in the intercalary heterochromatin on each chromosome, but not in the pericentromeric region (Staginnus et al. 1999, 2010). A similar distribution has been reported for the CaTy sequence, which shares homology with members of the Ty1/Copia-like element family (Staginnus et al. 2010). Only weak signal was obtained using a probe based on a chickpea LINE-like element (Staginnus et al. 2010).

FISH probes based on low or single copy sequences have been deployed in a number of plant species (Jiang et al. 1995; Lapitan et al. 1997; Zhang et al. 2004; Idziak et al. 2014). Zatloukalová et al. (2011) prepared a partial bacterial artificial chromosome library from desi chickpea genomic DNA and recovered five clones which hybridized to a single locus. One of the loci mapped to a subtelomeric region on the short arm of chromosome A, two to a subtelomeric region on each arm of chromosome B, one to one of the telomeres of chromosome E, and the last to a telomeric region on chromosome H (Fig. 4.5).

Although the number of informative FISH probes is not extensive, they are sufficient to identify five chromosomes in the karyotype. While this can provide opportunities to follow chromosome behavior during meiosis and to compare the karyotypes of cultivated and wild chickpea accessions, there is a need to elaborate additional cytogenetic markers. A possible option is to use cDNAs, since these have been successfully deployed in both barley (Karafiátová et al. 2013) and wheat (Danilova et al. 2014). The acquisition of the genome sequence means that, as has been pioneered in barley (Aliyeva-Schorr et al. 2015), it is now possible to identify in silico sequences suitable as FISH probes.

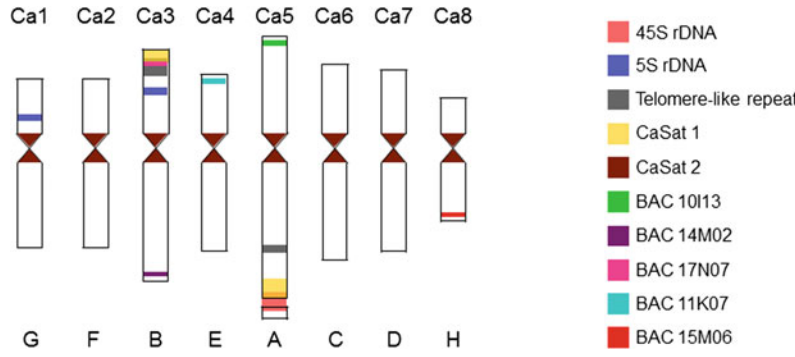

Fig. 4.5 Idiogram of *C. arietinum* desi type created using data on chromosome length by Ruperao et al. (2014) and location of a set of DNA sequences which were mapped to chromosomes using FISH (Zatloukalová et al. 2011 and unpublished data)

4.8 Chromosomal Organization at the Molecular Level

Thanks to the development of high-throughput sequencing, partial genome sequences of both desi (38.48%) and kabuli (39.37%) chickpea have been acquired (Jain et al. 2013; Varshney et al. 2013; Parween et al. 2015). The assembly of a whole genome sequence is highly revealing of chromosomal organization at the molecular level and allows for comparisons to be made of chromosome structure both within and between species (Paterson et al. 2009; Schatz et al. 2014; Schnable et al. 2009; Thiel et al. 2009). As in other plant species, the chickpea genome harbors a significant proportion of repetitive DNA, some of which is present in the form of an extended region of tandemly arranged repeats. As also suggested by the cytogenetic detection of heterochromatin (Staginnus et al. 1999, 2010; Zatloukalová et al. 2011), the centromeric and pericentromeric regions are particularly repeat-rich (especially with respect to the CaSat 2 element) and gene-poor. Parween et al. (2015) showed that the mean frequency of recombination in the pericentromeric region of desi is some ninefold lower than in more euchromatin-rich regions. Gene density across the desi pseudomolecules averaged 7.07 per 100 Kbp, about double the density (3.73 per 100 Kbp) present in unanchored scaffolds, implying that the latter sequences harbor a high proportion of repetitive DNA. The current desi and kabuli assemblies represent only 24–55% of each of the eight chromosomes, and the most distal and subtelomeric regions are mostly absent (Parween et al. 2015). Thus, it is not possible as yet to draw conclusions regarding gene density and repetitive DNA content along the full length of any of the chickpea chromosomes in the way that has been achieved in rice (Goff et al. 2002) and *Arabidopsis thalianan* (Schneeberger et al. 2011), for example, and even for one of the large chromosomes of wheat (Choulet et al. 2014). Nevertheless, the indications are that the desi and kabuli genomes are highly similar to one another. Ruperao et al. (2014) have suggested that apparent differences between the two assemblies are an artifact arising from the gappiness of the sequences. Clearly, a higher quality reference genome assembly will be needed to elaborate a more precise picture of chromosome organization at the molecular level.

4.9 Flow Cytogenetics

Flow cytometry can be highly informative with respect to chromosome size and structure (Kubaláková et al. 2003; Molnár et al. 2011; Ma et al. 2013). It supports physical mapping and whole genome sequencing, especially in the context of large genome species (Cviková et al. 2015; Raats et al. 2013; Ruperao et al. 2014; Mayer et al. 2014). Vláčilová et al. (2002) have described a protocol to synchronize cell cycle and thereby to accumulate chromosomes at mitotic metaphase in chickpea root tips and have exploited it to prepare liquid suspensions of intact chromosomes suitable for flow cytometry. The resulting flow karyotype of kabuli type comprised eight peaks, five of which were assignable using FISH to chromosomes A–C, G, and H. The other three peaks represented chromosomes D, E, and F. The purity of the single chromosome flow-sorted fractions ranged from 68% (chromosome C) to 100% (chromosomes B and H). Applying PCR assays targeting microsatellite loci confirmed that chromosome H was equivalent to linkage group LG8, marking the first step toward integrating the chickpea cytogenetic and genetic maps. When Zatloukalová et al. (2011) flow karyotyped the desi type, both the number and positions of the peaks differed from those forming the kabuli-type flow karyotype (Vláčilová et al. 2002): Here, only six peaks were observed. This difference implied that the two genomes were distinct from one another, at least with respect to their AT/GC content, in contradiction to the conclusion reached from an analysis of the partial genome assemblies that the two genomes are highly similar (see previous section). However, the difference is in line with the suggestions of Ohri and Pal (1991) and Kordi et al. (2006), which was based on DNA amount. The lack of agreement between the kabuli- and desi-type flow

karyotypes has recently been confirmed by Ruperao et al. (2014) and is illustrated in Fig. 4.6. Of the six peaks forming the desi-type flow karyotype, four were assigned using FISH to chromosomes A, B, E, and H, and each of the other two peaks was a mixture (one of chromosomes C and D, and the other of chromosomes F and G). The purity of the flow-sorted fractions involving a single chromosome varied from 88% (chromosome A) to 98% (chromosome H). PCR-based microsatellite assays confirmed that chromosome A is equivalent to LG5, B to LG3, E to LG4, and H to LG8. Similarly, it was concluded that chromosome F is equivalent to one of LG1 and LG2, and chromosome G to the other; while chromosomes is equivalent to one of LG6 or LG7, and chromosome D to the other (Table 4.1).

Flow-sorted chromosomes are also useful as a means of validating genome sequence assemblies. Purified preparations of desi-type chromosomes A, B, and H, as well as A–C and F–H of the kabuli-type chromosomes were used by Ruperao et al. (2014) as a template for Illumina-based sequencing. When compared to the desi assembled pseudomolecules (Jain et al. 2013), some large-scale misassignations became apparent, while in the kabuli assembly (Varshney et al. 2013), a number of short defined regions were shown to have been misplaced. Thanks to the recent development of a procedure for sequencing an individual flow-sorted chromosome (Cápal et al. 2015), it has now become possible to obtain sequence from a unique chromosome, although the identity of the sequenced chromosome cannot be known a priori. A further application of flow-sorted chickpea chromosomes has been as a target for FISH. When mounted on a microscope slide, a flow-sorted preparation typically comprises thousands of chromosomes at a high level of purity and free of cell and tissue debris, which improves the robustness of the FISH assay (Vláčilová et al. 2002; Zatloukalová et al. 2011).

4.10 Induced Polyploidy

Many crop species are polyploid (Zeven 1979; Li et al. 2015); although most are allopolyploid (e.g., wheat and cotton), a few are autopolyploid

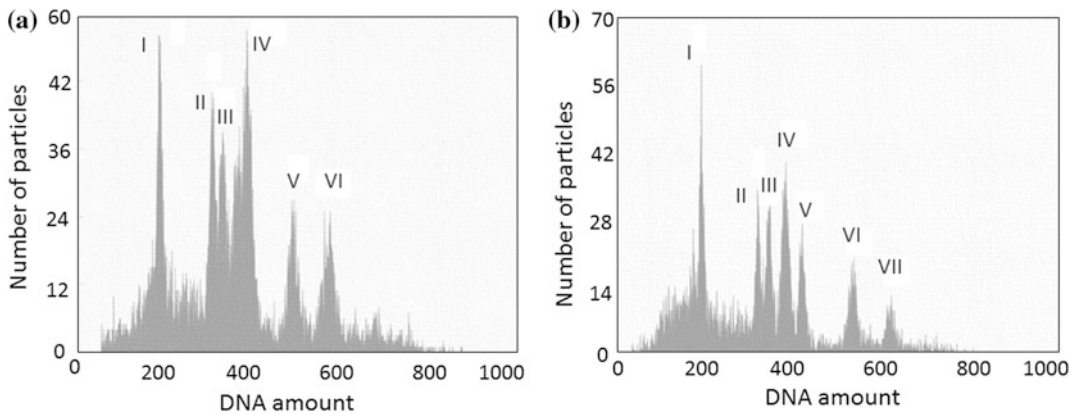

Fig. 4.6 Flow karyotype of (**a**) desi- and (**b**) kabuli-type chickpea. Liquid suspensions of mitotic metaphase chromosomes were stained by DAPI, and their relative fluorescence was analyzed using flow cytometry. Note differences in the number and position of chromosome peaks between the two chickpea types. In desi type, the flow karyotype comprises six peaks. Four peaks *I*, *III*, *V*, and *VI* represent chromosomes H, E, B, and A, respectively. Two composite peaks *II* and *IV* represent chromosomes F–G and C–D, respectively. In kabuli type, seven peaks could be resolved. Six peaks *I*, *II*, *III*, *V*, *VI*, and *VII* were assigned to chromosomes H, G, F, C, B, and A. Remaining composite peak *IV* represents chromosomes D–E

(potato and alfalfa). As increasing the ploidy level can be accompanied by improved plant performance (Ramsey and Ramsey 2014; Renny-Byfield and Wendel 2014), numerous attempts have been made to artificially induce autopolyploidy in a diploid crop species (Kinoshita and Takahashi 1969; Armstrong 1981). Sohoo et al. (1970) generated autotetraploid *C. arietinum* by treating the seedling apical meristem of both kabuli and desi types with colchicine. Although chromosome pairing at meiotic metaphase was dominated by bivalents, seed set in the autotetraploids was only about 30% that achieved in the diploids. Nevertheless, compared to their diploid progenitor, the autotetraploids did develop stronger and deeper roots, tougher stems, thicker pods, and bigger seeds. On the other hand, their germination was slow, and because of their reduced fertility, their grain yield was compromised (Sohoo et al. 1970; Pundir et al. 1983). In some induced autotetraploids, selection in subsequent generations has been able to restore fertility (Stebbins 1950), but the literature does not report any attempt to pursue this strategy in chickpea. The ability to reduce the ploidy level from diploid to haploid has been exploited as a means to rapidly fix a genotype via subsequent chromosome doubling, an approach which has been commercially exploited in a number of crop breeding programs, notably in barley (Forster et al. 2007), rice (Jiang et al. 2014) and eggplant (Rotino 2016). Haploids can be induced from either the microspore (androgenesis) or the megaspore (gynogenesis). The former approach typically relies on the in vitro culture of immature anthers. A first attempt to develop in vitro anther culture in chickpea was reported by Khan and Gosh (1983), which was followed by improvements in procedures to promote somatic embryogenesis and regeneration (Altaf and Ahmad 1986; Bajal and Gosal 1987; Huda et al. 2001; Vessal et al. 2002). Full protocols for the production of doubled haploid lines via androgenesis have been documented by Grewal et al. (2009) and Panchangam et al. (2014).

4.11 Wide Hybridization

There has been continued interest in the potential of wide hybridization as a means to improve chickpea. Targets for introgression have included disease resistance, stress tolerance, yield potential, and end-use quality. Post-fertilization incompatibility barriers are responsible for the relatively poor rate of success in producing wide hybrids. These include the presence of translocation differences between the parental genomes, leading to meiotic irregularities and a subsequent loss of fertility; cytoplasmic incompatibility; chromosome elimination and loss; excessive seed dormancy; and hybrid breakdown (Bassiri et al. 1987; Stamigna et al. 2000; Ahmad and Slinkard 2004). Although the fertilization process itself is relatively unhindered, the hybrid embryo often aborts within a few days. Attempts to deploy embryo rescue to circumvent this problem have not met with a great deal of success, and levels of efficiency are low (Verma et al. 1995; van Dorrestain et al. 1998; Mallikarjuna 1999).

While no published examples of a successful hybrid between chickpea and one of the perennial *Cicer* species exist, hybrids with several of the annual ones have been attempted (Croser et al. 2003). Hybrids are formable between *C. arietinum* and either *C. reticulatum* or *C. echinospermum* (the two species most closely related to the cultivated type), but their fertility is variable (Ladizinski and Adler 1976b; Singh and Ocampo 1993). If *C. arietinum* × *C. echinospermum* are highly sterile, *C. arietinum* × *C. reticulatum* F_1s are fertile and their meiosis is relatively regular, what tends to support the notion that *C. reticulatum* is the progenitor of the cultivated form (Ladizinski and Adler 1976b). The occasional meiotic irregularities observed in F_1 pollen mother cells comprise univalents and quadrivalents. According to Jaiswal et al. (1987), these hybrids flower early, have a high yield potential, and are better able to tolerate low temperatures than *C. arietinum*. The level of crossability between *C. arietinum* and *C. echinospermum* is low; the plants develop normally,

form six bivalents and two quadrivalents at meiosis, and are only partially fertile (Ladizinski and Adler 1976b). The presence of a quadrivalent suggests that the chromosomes involved have suffered a reciprocal translocation. A few interesting introgression events have been identified among the offspring of these two wide hybrids (Jaiswal et al. 1987; Singh and Ocampo 1993). A number of attempts to use either *C. bijungum* or *C. pinnatifidum* as a parent have failed (Singh et al. 1994, 1999; Verma 1990). However, the *C. arietinum* × C. *judaicum* hybrid was feasible (Verna et al. 1995); the resulting plants formed a high number of branches and pods and yielded well (Singh et al. 1994; Verma et al. 1995). In contrast, Ladizinski and Adler (1976b) did succeed in crossing *C. arietinum* with each of *C. judaicum*, *C. pinnatifidum*, and *C. bijungum*; meiotic pairing in each of these hybrids comprised mostly bivalents, with rare univalents, but the plants were all sterile. Recently, Abbo et al. (2011) described successful cross between annual *C. cuneatum* and perennial *C. canariense* with 50% pollen fertility and intermediate look of hybrid plants.

The outcomes of wide hybridization experiments led Ladizinski and Adler (1976b) to assign each of the annual *Cicer* species as a member of either the crop's primary genepool (*C. reticulatum*), its secondary genepool (*C. echinospernum*), or its tertiary genepool (*C. judaicum*, *C. pinnatifidum*, *C. bijungum*). The updated scheme suggested by Croser et al. (2003) matches the set of phylogenetic relationships derived by Buhariwalla et al. (2005) from a SNP-based analysis of genotypic diversity. Based on hybridization, Ladizinski and Adler (1976a, b) assigned all annual Cicer species to three crossability groups according to the classical definition as proposed by Harlan and de Wet (1971). More recently, this system was revised by Croser et al. (2003) Newly, primary genepool comprises *C. arietinum* and *C. reticulatum*, secondary genepool *C. echinospernum* only, and its tertiary genepool all remaining annual (and probably all perennial) Cicer species. This grouping correlates with genetic diversity of wild annual Cicer species (Buhariwalla et al. 2005).

4.12 Conclusion

Progress in chickpea cytogenetics has been slower than in many of the agriculturally important crops. There remain major knowledge gaps regarding chromosome structure both in the cultivated form and in its near relatives within the genus *Cicer,* and whether chromosome organization differs between the various *Cicer* species is quite unknown. Meiotic chromosome behavior in wide hybrids and their offspring are at best sketchily described. A major advance in filling these gaps should follow from the acquisition of the chickpea genomic sequence, the development of molecular cytogenetics technology, and the use of flow cytometry to apportion the nuclear genome into its component chromosomes. The probability is that in the near future, the chickpea community will be in a position to better utilize the full range of genetic diversity present in the genepool and thereby to support the breeding of improved cultivars of chickpea.

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