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RESEARCH

# Distinct Subgroups of *Cicer echinospermum* Are Associated with Hybrid Sterility and Breakdown in Interspecific Crosses with Cultivated Chickpea

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## ABSTRACT

Crop wild relatives are a reservoir of phenotypic variation not present in the germplasm of cultivated species and thus have great potential for crop improvement. However, issues of genetic compatibility often interfere with effective utilization of crop wild relative taxa. Among chickpea (*Cicer arietinum* L.) crop wild relatives, *Cicer echinospermum* P.H. Davis is the sole species in the secondary genepool, being partially compatible with the primary genepool that is composed of the cultigen and its progenitor wild species *Cicer reticulatum* Ladizinksy. We report results from genetic studies among interspecific hybrids between cultivated chickpea and accessions from six recently identified wild *C. echinospermum* sites in southeastern Turkey, encompassing the known genetic diversity of the secondary genepool. Our studies indicate that both hybrid sterility and hybrid breakdown occur and are associated with distinct subgroups of *C. echinospermum*. Analysis of early-generation progenies suggests that both hybrid sterility and hybrid breakdown are conditioned by one to few genetic loci. These results clarify ambiguity in the nature of the hybridization barriers of reduced fertility in interspecific crossing of cultivated chickpea with *C. echinospermum* and should foster a more systematic and wider use of *C. echinospermum* for base broadening of cultivated chickpea.

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**Abbreviations:** CWR, crop wild relative; QTL(s), quantitative trait loci.

Chickpea (*Cicer arietinum* L.) is a component of many Mediterranean and semiarid subtropical crop rotation systems (Hossain et al., 1996; Whish et al., 2007; Chattopadhyay and Mohapatra 2015). Its capacity for biological nitrogen fixation is useful agronomically (López-Bellido et al., 1996; López-Bellido and López-Bellido, 2001), while its seed is consumed as an important component of the human diet and the haulm (stover) has utility as forage. In many regions of the world, its high protein, mineral, vitamin, and fiber levels are important for nutritional security. Chickpea is among the oldest of crops, being domesticated in the Fertile Crescent ~10,000 yr ago (Redden and Berger, 2007). Although this nutrient- and protein-rich crop is the second largest food grain legume (pulse) crop in the world in acreage and production (FAO database), its susceptibility to diseases and environmental conditions remains a challenge to greater productivity (Ghosh et al., 2013; Rubiales et al., 2015). The narrow genetic base of cultivated chickpea (Roorkiwal et al., 2014; Saxena et

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al., 2014a, 2014b; Bajaj et al., 2015; Van Oss et al., 2015) has been suggested to limit its capacity to tolerate stresses (Abbo et al., 2003). At least two bottlenecks underlie the loss of genetic diversity in cultivated chickpea relative to the immediate wild progenitor (*Cicer reticulatum* Ladizinksy), including the founder effect during domestication and the substitution of landraces by modern cultivated varieties (Abbo et al., 2003).

Crop wild relatives (CWRs), species from within the genus to which the cultigen belongs and closely related sister genera, are sources of diversity (Kumar and Dua, 2006; Kumar et al., 2011, Singh et al., 2013; Warschefsky et al., 2014) for broadening the genetic base of cultivated genepools (Hawkes, 1977; Doyle, 1988; Tanksley and McCouch, 1997). The most facile means to tap the diversity of CWRs is by interspecific hybridization in genetic crosses between cultivated germplasm and CWRs, with the use of in vitro embryo rescue and propagation as a more cumbersome and thus less desirable route. Recalcitrance in the development of interspecific hybrids with the cultigen and CWRs represents a significant biological barrier.

The genus *Cicer* has 43 species including nine annuals, with the remainder being perennials (Van der Maesen, 1987). Annual species include *C. arietinum* L. (domesticated chickpea), *C. reticulatum*, *Cicer echinospermum* P.H. Davis, *C. bijugum* K.H. Rech, *C. chorassanicum* M. Pop, *C. cuneatum* Hochst. Rich, *C. judaicum* Boiss., *C. pinnatifidum* Jaub. et Spech, and *C. yamashitae* Kitamura. Several wild *Cicer* species have been identified as a rich source of alleles desirable for yield component traits such as seed per plant, harvest index (Cengiz Toker, personal communication, 2017), and resistance towards environmental stresses (Van der Maesen and Pundir, 1984; Singh et al., 1994, 1998, 2005; Singh and Ocampo, 1997; Croser et al., 2003; Rao et al., 2003; Toker, 2005; Sandhu et al., 2006; Sharma et al., 2006; Mallikarjuna et al., 2007; Toker et al., 2007; Kaur et al., 2010, 2013; Tekin et al., 2017). Some wild species such as *C. bijugum, C. pinnatifidum*, *C. reticulatum*, and *C. echinospermum* are resistant to multiple stress conditions (Kumar et al. 2011). For example, C. *echinospermum* harbors stress tolerance traits for cold, pod borer (*Helicoverpa armigera* Hübner), and Phytophthora root rot [*Phytophthora citrophthora* (R.E. Sm. & E.H. Sm. Leonian] (Singh et al., 1990; Kaur et al., 1999; Sharma, 2004; Knights et al., 2008) that mitigate reduced productivity under stress conditions.

*Cicer reticulatum* and *C. echinospermum* are the most closely related CWRs of the cultigen *C. arietinum*. On the basis of morphological characteristics, notably in seed coat color and features, and genetic crossability and cytogenetic studies, *C. reticulatum* was proposed as the progenitor of the cultigen, with *C. echnospermum* as the sole species in the secondary genepool (Ladizinsky and Adler, 1976). *Cicer reticulatum* and *C. echinospermum* meet the criteria of Harlan and De Wet (1971) for the primary and secondary genepools, respectively, of cultivated chickpea. This affiliation is supported by subsequent cytogenetic studies wherein distinct C-banded heterochromatin content, chromosome banding patterns, satellite location, and karyotype asymmetry among the species were observed (Ocampo et al., 1992; Tayyar et al., 1994; Galasso et al., 1996). Further studies used a range of molecular markers based on seed storage proteins, random amplified polymorphic DNA, amplified fragment length polymorphisms, and microsatellites (Kazan and Muehlbauer, 1991; Ahmad et al., 1992; Labdi et al., 1996; Tayyar and Waines, 1996; Ahmad, 1999; Choumane et al., 2000; Sudupak et al., 2002; Shan et al., 2005; Van der Maesen et al., 2007). Based on these studies, *C. echinospermum* in now recognized as a distinct and more distant species from the progenitor *C. reticulatum*.

The ability to obtain interspecific hybrids with *C. reticulatum* and *C. echinospermum* has led to their more intensive use for introgression breeding (Pundir and Mengesha, 1995; Iruela et al., 2002; Sudupak et al., 2002; Nguyen et al., 2004; Cingilli et al., 2005; Shan et al., 2005; Bharadwaj et al., 2011). To date, gene introgressions from CWRs into cultivated chickpea have been reported for quality traits and biotic and abiotic stresses (Singh et al., 1994, 2005; Singh and Ocampo, 1997). Most of these introgressions have been with *C. reticulatum*, the crop's immediate progenitor (Hajjar and Hodgkin, 2007; Kumar et al., 2011), which is fully cross-compatible with the cultigen. Cross-compatibility of *C. echinospermum* with cultivated chickpea is low, with sterility being encountered in hybrids in the first filial generation (Ladizinsky and Adler, 1976; Singh et al., 1991) or in early segregating generations (Kazan and Muehlbauer, 1991). Improved success has been noted based on the particular *C. echinospermum* used and, to a lesser extent, on the cultivated genotype serving as the female parent (Singh and Ocampo, 1993; Pundir and Mengesha, 1995). Despite the existence of partial sterility in progenies of *C. arietinum* × *C. echinospermum*, several interspecific hybridization populations have been produced, with selections made for improved seed yield (Singh and Ocampo, 1997) and disease tolerances for Ascochyta blight [*Ascochyta rabiei* (Passerini) Labrousse] (Collard et al., 2003), Phytophthora root rot (Knights et al., 2008), and root nematodes [*Pratylenchus thornei* Sher & Allen, *P. neglectus* (Rensch, 1924) Filipjev Schuurmans & Stekhoven] (Thompson et al., 2011).

To discern the stages where barriers to interspecific hybridization operate, pollen growth in vivo on pistils and early embryonic development were examined in crosses between *C. arietinum* and different annual CWR species (Ahmad et al., 1988; Ahmad and Slinkard, 2004). In these studies, although zygotic stages were observed in cross-pollinated flowers with all CWRs, only those from the primary and secondary genepool species (*C. reticulatum*

and *C. echinospermum*, respectively) continued to progress to the subsequent embryo stage. This suggests that the postzygotic stage is a significant barrier to interspecific hybridization beyond the secondary genepool (i.e., with CWRs from the tertiary genepool).

Although several researchers have reported outcomes of interspecific hybridization of *C. arietinum* with *C. echinospermum* (Ladizinsky and Adler, 1976; Kazan and Muehlbauer, 1991; Pundir and Mengesha, 1995), there is ambiguity with respect to when incompatibility or sterility is manifested. Recent systematic collections of *C. echinospermum* in 2013 (Eric von Wettberg and Douglas Cook, personal communication, 2017) and in subsequent years (Jens Berger, personal communication, 2017) substantially improve germplasm for this species, along with improved characterization of its genome and ecology. Use of *C. echinospermum* for crop improvement would benefit from a better understanding of fertility barriers in interspecific crosses with the crop.

Here, we present results of our investigations on the cross compatibility between cultivated chickpea, *C. arietinum*, and *C. echinospermum*. In contrast with historical work with CWRs that typically evaluated one to few accessions per species, we describe results on fertility in F1 hybrids and their derivative offspring with six different *C. echinospermum* accessions that together represent the genetic diversity of the wild species. By clarifying the type and distribution of variation among *C. echinospermum* and the apparent simple genetic inheritance for sterility barriers, our results should facilitate the use of this secondary genepool CWR in crop breeding to broaden the genetic base of chickpea.

# MATERIALS AND METHODS

## Plant Materials

Geographic locations of sites from where *C. echinospermum* were collected in 2013 and the focus of this study are presented in Fig. 1. Information on wild *C. echinospermum* genotypes used in this study is presented in Supplemental Table S1.

## Genetic Analysis and Plant Handling

Genetic crossing was done with glasshouse- or field-grown plants. Unless otherwise noted, crossing was done with cultivated genotypes as the female (pollen recipient) parents and with wild accessions serving as male parents (pollen donor). For emasculation, floral buds, before full anthesis, were gently opened with fine-tipped forceps and all the anthers removed, with care taken to avoid touching of the style and stigma. For cross-pollination, pollen from freshly tripped flowers at full anthesis of wild genotypes was harvested onto the tips of forceps and applied by brushing gently onto the stigmatic surface of recipient buds. After pollination, pistils were covered with the subtending wing and keel petals and buds marked with jeweler's tags. Seed from crosses were harvested after full physiological maturity of pods.

Seeds obtained from crosses were germinated and grown individually along with those of the parental wild and cultivated genotypes, which were grown as references. True wild-cultivated hybrids exhibited phenotypes that were intermediate to those of the cultivated and wild parents, including a semi-erect growth habit, greater numbers of lateral branches than cultivated parents, and a protracted duration of pod setting. These traits contrasted readily with the erect, compact, and defined plant maturity duration of the cultivated parents, allowing for hybrids to be distinguished readily from self-fertilized seed of unsuccessful crosses. In addition, on entry into the reproductive phase, known recessively inherited traits in cultivated parents for white flower color in 'kabuli' cultivars (CDC Leader, Gokce) and super-early flowering in ICCV 96029 served to distinguish true crosses from self-fertilized seeds. Conversely, the occurrence in putative F1 hybrids of traits conditioned by dominant alleles in single genes, such as purple flower color, served to identify seed of successful cross-fertilization from inadvertently self-pollinated individuals.

## Pollen Viability Assays

Two different assays were used to assess viability of pollen. For assessing pollen viability per se, the colorimetric staining protocol of Peterson et al. (2010, with minor adjustments) was used. Flower buds with mature, preanthesis, nondehiscent anthers were collected from greenhouse-grown plants and fixed in Carnoy's fixative (6 alcohol: 3 chloroform: 1 acetic acid) for 2 h to remove oils and clear anther walls. Each bud was then dab dried using absorbent paper while placed on a slide. Two to four drops of the final stain solution, prepared according to Peterson et al. (2010), were applied just before the buds completely dried. The buds were then dissected with fine forceps to release the anthers and pollen, with the rest of the debris carefully removed under a dissecting microscope. Each sample slide was then heated up over a moderate alcohol burner, by moving it slowly across the flame for ~30 s and the stain solution brought to near boiling. The samples were then allowed to cool and stain for 30 to 60 min before imaging. Imaging was done on a dissecting microscope (Olympus, Model SZX16), after placing a cover slip on the sample, for viable pollen (stained magenta-red) versus inviable pollen (stained blue green).

As another measure of pollen functional integrity beyond viability, we assessed the ability of pollen to germinate and grow in vitro using the procedure of Shivanna et al. (1997). Pollen or entire anthers that were crushed gently to liberate pollen grains were obtained from greenhouse-grown plants. Pollen or anthers were placed and grown on modified Brewbecker and Quacks broth consisting of 2.5% sucrose, 10% PEG, 100 mg Boric Acid, 300 mg $Ca(NO_3)_2$, 200 mg $MgSO_4$, and 100 mg $KNO_4$ in 1 L mix adjusted to pH 7. The broth was preincubated at room temperature. A drop of the broth (~20 μL) was placed on a concave microscopic slide. Fully open flowers, from the anthesis developmental stage, were collected from each plant and anthers shaken gently, releasing the pollen onto the meniscus of the medium on the slide. Each slide was then incubated in a foil-wrapped, parafilm-sealed Petri dish over moist filter paper for 90 min and observed for tube growth under a dissecting microscope.

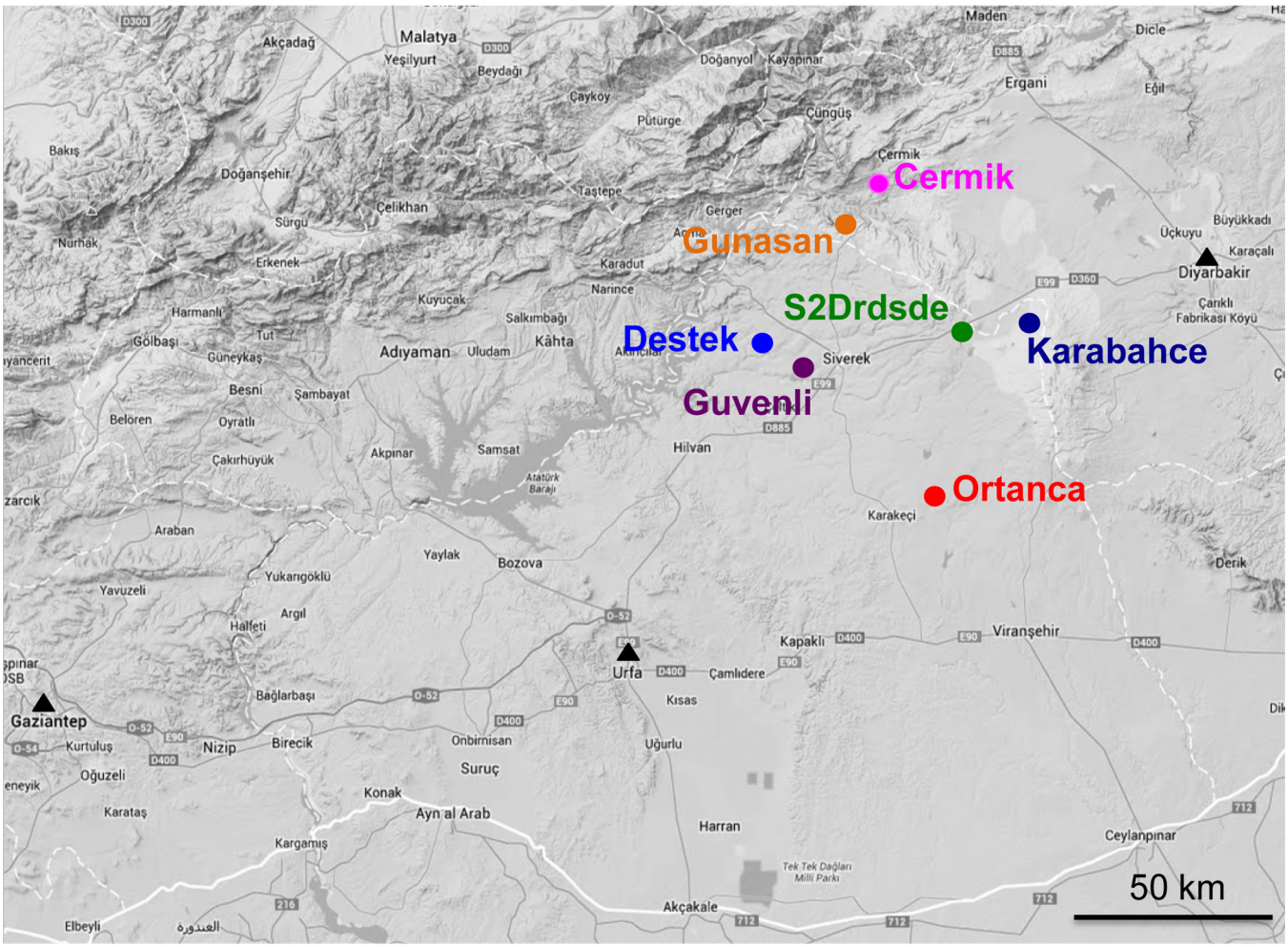


Fig. 1. Locations of *C. echinospermum* wild accessions collected in 2013 in southeastern Anatolia are marked by closed circles. Filled black triangles mark from left to right: Gaziantep, Sanliurfa, and Diyarbakir, the major metropolitan areas in the region. The undulating white line at the bottom of the image marks the boundary between Turkey and Syria.

## RESULTS

### Reduced Representation of Extant Diversity in *C. echinospermum*

Collection of CWRs from southeastern Turkey in summer 2013 yielded a total of 80 *C. echinospermum* individuals, sampled nondestructively from six wild field sites (Fig. 1). Single-seed descent lineages were grown ex situ to immortalize this collection as curated germplasm accessions (Supplemental Table S1). Molecular diversity analysis of data obtained from restriction site-associated genotyping by sequencing of these 80 new *C. echinospermum* accessions indicated that accession-to-accession variation within a collection site was of lower magnitude when compared with diversity of accessions from across the six sites (Eric von Wettberg and Douglas Cook, personal communication, 2017), reflecting discrete local genetic structure and predominantly inbreeding characteristics. Historically collected *C. echinospermum* nested within a portion of the new collection. For the subsequent studies of genetic compatibility, we selected a single genotype from each of the six wild collection sites to represent the diversity among the larger set of 80 *C. echinospermum* accessions. Such a reduction made it feasible to examine intercrossing between each of the six wild field sites and multiple *C. arietinum* cultivars from major chickpea production areas: India, Turkey, and North America.

### Variation among *C. echinospermum* for Self-Fertilized Seed Set in $F_1$ Hybrids with Cultivars

Accessions from the six *C. echinospermum* sites were used in crosses to four different chickpea cultivars: ICCV 96029 and CDC Consul of the 'desi' type, and CDC Leader and Gokce of the 'kabuli' type. Due to the ease of crossing into the cultigen, relative to crossing into wild genotypes, we initially performed single-direction crosses with the cultivated genotype as the female parent. We obtained $F_1$ seed from crosses into the cultigens for all biparental combinations, except for some combinations with the cultivar 'Gokce' that were not attempted. True hybrid $F_1$ individuals were readily distinguished from failed crosses according to the number of lateral branches and growth habit of hybrids, which were intermediate between wild

*C. echinospermum* and *C. arietinum* parents. Initially, we confirmed the relationship between phenotypic markers and hybridity using molecular markers, whereas for subsequent work, we relied solely on morphological criteria to distinguish true hybrids from self-pollinated progeny. Although the $F_1$ hybrids from different *C. echinospermum* genotypes were similar to one another during the vegetative phase, hybrids from crosses with genotypes from Cermik, Destek, and Ortanca were sterile, whereas hybrids from crosses with genotypes from Gunasan and S2Drdsde exhibited high fertility, and crosses to the Karabahce genotype were of variable fertility, depending on the genotype of the cultivated recipient (Table 1). The number of $F_2$ seed also varied depending on the cultivation conditions, with those grown in greenhouse pot cultures yielding <200 $F_2$ seed per $F_1$ plant, and those from vegetatively propagated stem cuttings or in field cultivation yielding >400 $F_2$ seed per $F_1$ plant. These yields are comparable with the range of $F_2$ seed per $F_1$ plant obtained under similarly grown $F_1$s of interspecific hybrids with *C. reticulatum* (data not shown).

On the basis of $F_1$ fertility (Table 1), we classified the six *C. echinospermum* genotypes into two distinct groups: Group I consisted of accessions from Cermik, Destek, and Ortanca field sites for which $F_1$s failed to yield $F_2$ seed (e.g., hybrid sterility), and Group II was composed of accessions from Gunasan, Karabahce, and S2Drdsde whose $F_1$s yielded fertile $F_2$ seeds but that segregated for sterility (e.g., hybrid breakdown) in later generations. To further examine the mechanisms underlying hybrid sterility in Group I and hybrid breakdown in Group II, we focused our efforts on characterizing the progenies of the six *C. echinospermum* genotypes crossed with the cultivar ICCV 96029.

## Pollen Inviability in $F_1$ Hybrids with *C. echinospermum* Group I Genotypes

$F_1$ progeny from Group I genotypes crossed into ICCV 96029 produced floral organs in buds and developing flowers that were morphologically similar to those of flowers of the parental genotypes and of other hybrids that produce viable self-fertilized seed, with sepals, petals, anthers, and stamens being indistinguishable from those of normal, self-fertile flowers. However, at full and postanthesis stages, Group I hybrids lacked dehiscent pollen grains that typify normal anthers at full maturity. Manual crushing of anthers from flowers at and after full anthesis from these hybrids failed to liberate dehiscent pollen, suggesting the absence of functional pollen as a potential basis for the absence of self-fertilized seed in these genotypes.

Pollen viability of the cultivated parent ICCV96029, the Group I wild donor Ortanca, and derived $F_1$ hybrids was evaluated in two assays: a staining assay that distinguishes viable from inviable pollen (Peterson et al., 2010), and an in vitro pollen germination test (Shivanna et al., 1997). Staining of isolated anthers from the parental genotypes yielded magenta-stained pollen grains (Fig. 2, top row), a characteristic of viable pollen (Peterson et al., 2010). By contrast, anthers from $F_1$ hybrids were predominantly stained blue (Fig. 2, second row) and very infrequently magenta, which is indicative of a high rate of inviable pollen. Similarly, in the in vitro pollen growth assay, pollen grains from the parents germinated and yielded elongated pollen tubes (Fig. 2, third row), whereas pollen of $F_1$ hybrids failed to germinate (Fig. 2, bottom row). Anthers and pollen from $F_1$ hybrids involving the other two Group I *C. echinospermum* genotypes, Cermik and Destek, yielded similar results (data not shown). Taken together, the pollen viability staining and in vitro pollen germination data indicate that the failure to obtain self-fertilized seed in the $F_1$ hybrids of *C. arietinum* and *C. echinospermum* Group I genotypes is a consequence of inviable pollen.

To test whether the female organs (gynoecium) may also be altered in the hybrids, we performed artificial hybridization of $F_1$ flowers with pollen from either of the cultivated ICCV 96029 or the *C. echinospermum* parents. In contrast with the absence of pod and seed from self-fertilization in the $F_1$ hybrids, artificial pollination with pollen from either the cultivated or wild parents produced viable $BC_1$-$F_1$ seed and progeny. Reciprocal crosses involving the Group I genotype Ortanca as female and the cultivated parent ICCV 96029 as male yielded similar results (Table 2), with $F_1$ progeny that were vigorous but infertile and infertility rescued by backcrossing with pollen from either the ICCV 96029 or Ortanca parent. Thus, hybrid sterility as a consequence of pollen inviability is independent of the directionality of the initial cross.

**Table 1. Fecundity (the number of seed produced) of $F_1$ hybrids of six representative *C. echinospermum* accessions in crosses with four cultivars of *C. arietinum*. The number of $F_2$ seeds obtained from $F_1$ hybrids for each of the wild-cultigen combinations is listed. Fecundity yields of <200 $F_2$ seed per $F_1$ plant were from plants grown in greenhouse or field conditions, whereas those >400 seed per $F_1$ plant were from vegetatively amplified stem cuttings of $F_1$ individuals.**

| | Male parent, *C. echinospermum* | | | | | |
|---|---|---|---|---|---|---|
| Female parent | Cermik | Destek | Ortanca | Gunasan | Karabahce | S2Drdsde |
| ICCV 96029 ('desi') | 0 | 0 | 0 | 141 | 68 | 165 |
| CDC Consul ('desi') | 0 | 0 | 0 | 470 | 0 | 403 |
| CDC Leader ('kabuli') | 0 | 0 | 0 | 446 | 0 | 449 |
| Gokce ('kabuli') | n.d.† | n.d. | n.d. | n.d. | 70 | n.d. |

† n.d., not determined; these crossing combinations were not conducted.

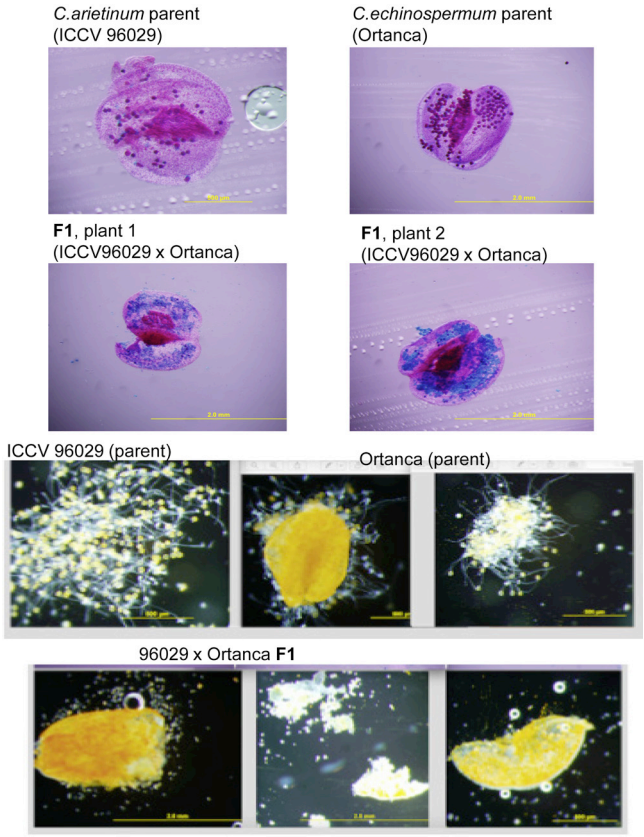


Fig. 2. Pollen viability (top two rows) and germination (bottom two rows) among parental genotypes *C. arietinum* ICCV 96029, *C. echinospermum* Ortanca, and their $F_1$ hybrid (ICCV 96029 × Ortan). Viable pollen stain purple are shown in the top row, and nonviable pollen stain blue are shown in the second row. Punctuated bright dots in bottom two rows are individual pollen grains. In this assay, pollen tubes from germinated pollen are visible as filamentous structures emanating from individual pollen grains in the parental genotypes (third row) but not in the $F_1$s (bottom row). Entire anthers or portions of anthers are shown in the top row middle images and in images of the bottom row.

## Inheritance of Self-Sterility in *C. echinospermum* Group I Interspecific Hybrids

The inheritance of hybrid sterility in Group I was examined in the progeny of $F_1$ hybrids (*C. echinospermum* Ortanca × the cultigen ICCV 96029) backcrossed crossed to either parent as the pollen donor. In total, we obtained 11 and 7 from backcrosses ($BC_1$-$F_1$) of $F_1$ hybrids to the cultigen ICCV 96029 or *C. echinospermum* genotype Ortanca, respectively (Table 3). Germination of these 18 $BC_1$-$F_1$ individuals yielded $BC_1$-$F_1$ progeny with vegetative phenotypes reflecting their backcross parentage. These $BC_1$-$F_1$ plants continued growth to the reproductive phase when flowers that appeared to be macroscopically normal were produced.

Upon flowering, all seven of the $BC_1$-$F_1$s derived from backcrossing to the wild Ortanca parent failed to set seed (Table 3). By contrast, of 11 $BC_1$-$F_1$s obtained in crosses to the cultigen ICCV 96029, six individuals failed to produce self-fertilized seed, whereas the remaining five individuals produced self-fertilized seed (Table 3). A chi-square test for goodness of fit with the observed numbers of self-fertile to self-infertile individuals among these 11 $BC_1$-$F_1$s to a 1:1 model was statistically supportive of a fit to this model (Table 3, $p = 0.763$).

The combined data for all crosses fit a classical model for cytoplasmic male sterility, with a negative interaction between the cultivated (female) cytoplasm and a dominant nuclear allele in the wild genome leading to nonviable pollen. Fertility is recovered at a 50% rate on backcrossing to the cultivated genome, which is the rate at which the wild allele is eliminated, but never recovered on backcrossing to the wild genome because both the cytoplasm of the cultigen and at least one allele from the wild genome is constantly present.

## Varying Self-Fertilized Seed Rates in $F_2$ and $F_3$ Generations of Interspecific Hybrids with Group II *C. echinospermum*

For Group II, the three *C. echinospermum* genotypes from Gunasan, Karabahce, and S2Drdsde, whose $F_1$ hybrids yielded $F_2$ seed in crosses with two or more cultivars (Table 1), we grew batches of $F_2$ seed to examine fecundity in the next generation. $F_2$ plants from these three *C. echinospermum* × ICCV 96029 populations all segregated for a similar range of vegetative phase traits (e.g., plant habit, branching, and days to the onset of flowering). Buds and flowers of these $F_2$ individuals were similar to those of the wild and cultivated parental genotypes and to $F_2$ derived from crosses of cultivated to *C. reticulatum*. In contrast with hybrid populations from *C. reticulatum*, all hybrid $F_2$ populations derived from Group II *C. echniospermum* parents displayed a gradient of fertility (Fig. 3). A portion of $F_2$ individuals in each population exhibited normal, high rates of pod setting and seed maturation. Fertility in the remaining $F_2$ individuals of these populations varied. In some individuals, pod development initiated but failed to be sustained beyond 1 to 2 wk, yielding small empty pods that fell away. In other $F_2$ individuals, pod development continued longer to sizes of normal seed-filled pods, but at maturity, such pods contained only incompletely developed shriveled seeds. Sterile plants tended to grow vigorously, with dense branching and a long flowering period.

**Table 2. Number of $F_1$ individuals and their self-(in)fertility in reciprocal crosses between cultigen ICCV 96029 and Group I *C. echinospermum* genotype Ortanca.**

| Female parent | Male parent | Assayed individual's genotype | No. of individuals phenotyped | Self-fertility phenotype |
|---|---|---|---|---|
| (echi) Ortanca | (ari) ICCV 96029 | $F_1$ | 2 | All sterile |
| (ari) ICCV 96029 | (echi) Ortanca | $F_1$ | 6 | All sterile |

Table 3. Self-fertilized seed setting in $BC_1$-$F_1$ individuals from backcrosses of the $F_1$ hybrid of ICCV 96029 × Ortanca and its parental genotypes ICCV 96029 and Ortanca.

| Female parent | Male parent | Assayed individual's genotype | No. of individuals phenotyped | Self-(in)fertility phenotype |
|---|---|---|---|---|
| [ICCV 96029 × Ortanca]-$F_1$ (*C. arietinum* × *C. echinospermum*) | Ortanca (*C. echinospermum*) | $BC_1$-$F_1$ | 7 | All 7 self-sterile |
| [ICCV 96029 × Ortanca]-$F_1$ (*C. arietinum* × *C. echinospermum*) | ICCV 96029 (*C. arietinum*) | $BC_1$-$F_1$ | 11 | 5 self-fertile, 6 self-sterile† |

† Chi-square test of goodness of fit to 1:1 ratio. Chi-square sum = 0.091, $p$ = 0.763.

## Inheritance of Self-Fertility in Group II *C. echinospermum*

We measured the rates of fertility in the $F_2$ generation from tallies of $F_2$s that gave $F_2$:$F_3$ seed and those $F_2$s that failed to yield $F_3$ seeds. When analyzed individually by cross, and as an aggregate, the observed proportions of self-fertile to self-infertile individuals in the three $F_2$ populations were statistically indistinguishable from those expected with a 1:3 ratio (Table 4), suggesting that self-fertility in the three Group II *C. echinospermum* interspecific hybrids populations is controlled by single recessive genetic loci.

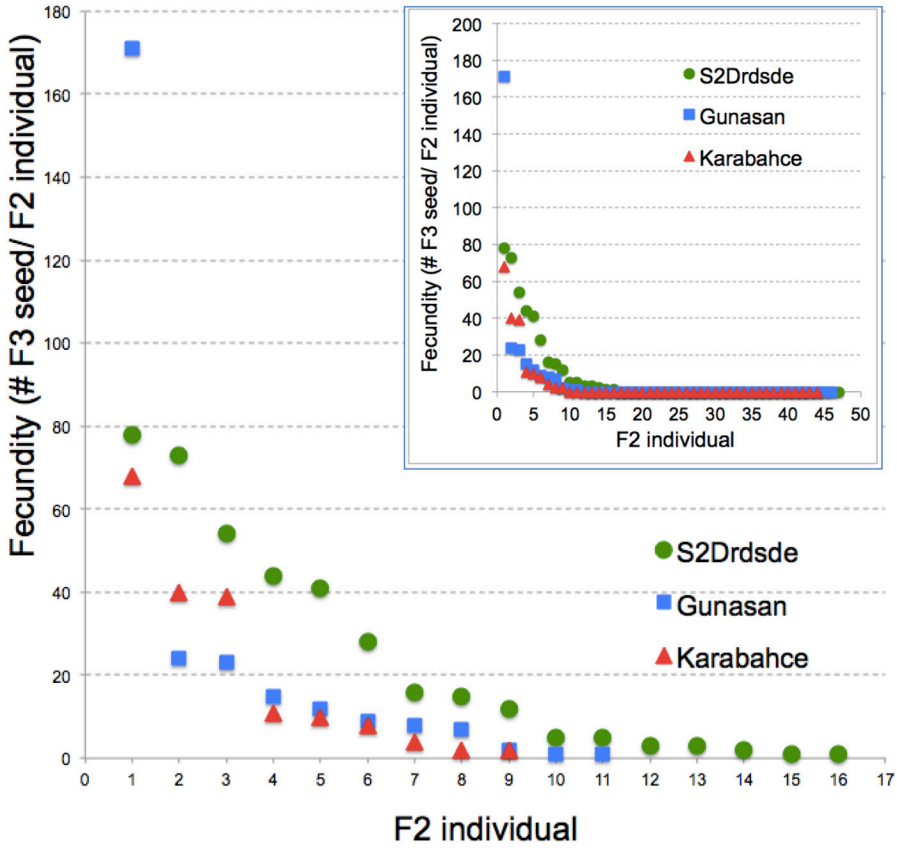


Fig. 3. Fecundity ($F_3$ seed yields) of $F_2$ individuals from crosses with cultivar ICCV 96029 and the three *C. echinospermum* Group II genotypes. The inset shows fecundity of all individuals analyzed, where a large proportion of individuals were self-sterile, yielding no $F_2$:$F_3$ seed, as reflected in the long tails of nil values along the *x*-axis. Fecundity of only self-fertile $F_2$ individuals of each population shown in the inset is presented in the main plot. To improve resolution among populations and individuals, linear values for fecundity shown in the inset are presented on a logarithmic scale (*y*-axis) in the main plot.

## DISCUSSION

Inviability, reduced vigor, or loss of fertility is commonly encountered in interspecific hybrids due to a range of mechanisms that ensure reproductive isolation in plants (Chen et al., 2016). Such reproductive isolation could be advantageous in some contexts, for example in natural systems where reduced fitness of interspecific helps to maintain species integrity, or in agricultural contexts to limit gene flow between crops and their weedy or wild relatives. However, reproductive isolation mechanisms can also be disadvantageous by limiting gene flow that would create novel genetic combination for natural selection to act on, and in the context of crop improvement by serving as a barrier to the use of crop wild relatives for beneficial traits absent from the cultigen's genepool.

Infertility of interspecific hybrids of cultivated chickpea and *C. echinospermum* was among the bases for the original recognition of *C. echinospermum* as a distinct species from *C. reticulatum*, the wild progenitor of chickpea, and the placement of *C. echinospermum* in the secondary genepool (Ladizinsky and Adler, 1976). However, since this seminal study four decades ago, results in subsequent studies involving crosses of the cultigen with *C. echinospermum* have been more varied (Kazan and Muehlbauer, 1991; Singh and Ocampo, 1993; Pundir and Mengesha, 1995). In some studies, complete sterility of hybrids was observed, in a manner similar to those of Ladizinsky and Adler (1976), whereas other studies report successful introgression of traits from *C. echinospermum* (Singh and Ocampo, 1997; Collard et al., 2003; Knights et al., 2008; Thompson et al., 2011). Thus, there is currently ambiguity in the ability of plant breeders to use *C. echinospermum* as a source of agronomically useful traits via interspecific hybridization. To clarify this ambiguity, in interspecific hybridization with *C. echinospermum*, we systematically examined *C. echinospermum* for crossability to the cultigen.

Table 4. Fertility among $F_2$ individuals from crosses with ICCV 96029 and Group II *C. echinospermum* genotypes that yield self-fertilized $F_2$ seed from $F_1$ hybrids. Values from a chi-square test for a single gene model wherein self-fertility is recessive and self-sterility is dominant are listed.

| CWR† parent | No. of $F_2$ plants | Self-fertile $F_2$ (yield $F_2$:$F_3$ seeds) | Self-infertile $F_2$ (no $F_2$:$F_3$ seeds) | Test ratio of self-fertile:self infertile | Chi-square test for 1:3 | |
|---|---|---|---|---|---|---|
| | | | | | Chi-square sum | *p* value |
| Gunasan | 46 | 11 | 35 | 1:3 | 0.029 | 0.865 |
| Karabahce | 44 | 9 | 35 | 1:3 | 0.485 | 0.486 |
| S2Drdsde | 47 | 16 | 31 | 1:3 | 2.050 | 0.152 |

† CWR, crop wild relative.

We focused our genetic studies on six representative accessions of *C. echinospermum* (one accession per site) collected recently in Southeastern Anatolia (Eric von Wettberg and Douglas Cook, personal communication, 2017) and four cultivars that together represent breeding programs in Turkey, India, and North America. The newly collected *C. echinospermum* derive from a systematic survey of wild populations, and where the genomes and collection site ecology are being characterized in ongoing research projects (Jens Berger, personal communication, 2017; Eric von Wettberg and Douglas Cook, personal communication, 2017). This collection encompasses and substantially expands the genetic diversity of previously collected *C. echinospermum* in international genebanks. Molecular diversity analysis (Eric von Wettberg and Douglas Cook, personal communication, 2017) indicates that accessions from within a collection site are highly similar, with the greatest extent of variation among collection sites. The predominantly self-pollinating nature of annual *Cicer* species, including *C. echinospermum*, the relatively large geographic distances (of several to many tens of kilometers) between the collection sites, and the patchy discontinuous distribution of wild *Cicer* might all contribute to such genetic differentiation. The observed pattern of discrete genetic groups and low levels of diversity within sites supports the logic of using single accessions to represent variation among the collected *C. echinospermum*. Importantly, use of single *C. echinospermum* accessions for each collection site made it feasible to examine (in)fertility in crosses and progenies from crosses of four elite cultivars representing the major chickpea production areas of Turkey, India, and North America.

We find that two types of fertility barriers operate in interspecific crosses between *C. echinospermum* and cultivated chickpea that manifest in distinct subgroups of *C. echinospermum*. Group I genotypes of *C. echinospermum* yield viable, morphologically normal hybrid individuals that fail to self-set seed, characteristics of the hybrid sterility type of barrier. Group II genotypes of *C. echinospermum* yield $F_1$ hybrids that are fully self-fertile but with variable rates of fertility in later generations, features that are reminiscent of hybrid breakdown. Additional studies are necessary to determine if the locus we identified among Group II *C. echinospermum* may be the genetic locus *Str/str* previously described (Kazan et al., 1993). An unresolved question is whether a combination of the two barrier types might also operate in tandem, wherein hybrid breakdown occurs in later generations of backcross-rescued progeny from *C. echinospermum* accessions from the Group I of hybrid sterility. This requires further analysis of later generations of backcross progenies that we have studied to date. Additional experiments are also needed to determine whether the same factors operate within the three different collection sites of each of the two subgroups, or if there is genetic heterogeneity for sterility within each of the defined groups. For example, preliminary results of quantitative trait loci (QTL) mapping for (in)fertility in the small scale $F_2$ populations (Table 4) yielded different QTLs in different populations of Group II (data not shown). This suggests potential diversity among different *C. echinospermum* sites within Group II for (in)fertility and underscores the need for additional, more detailed analysis.

Our current data suggest relatively simple genetic control in both hybrid sterility (Group I) and hybrid breakdown (Group II). In Group I, the patterns of complete self-sterility in $F_1$ hybrids, together with the absent fertility on backcross to the wild parent and a 1:1 ratio of fertility/infertility on backcross to the cultivated parent, are consistent with a single dominant nuclear locus from the wild parent that interacts negatively with a factor (presumably the mitochondrial genome) from the cultivated parent. This is reminiscent of classic cytoplasmic male sterility. By contrast, Group II hybrids were fully fertile and their $F_2$ progeny segregated for sterility at rates, consistent with a single recessive allele. However, the underlying genetic mechanisms may be more complex. For example, the high rate of reduced fertility in the $F_3$ generation of Group II hybrid populations (Fig. 3) suggests the involvement of additional genetic loci that modify or contribute to hybrid breakdown in Group II.

Our working hypothesis in conducting the reciprocal cross with Group I accession Ortanca (Table 2) was to assess whether cytoplasmic factors underlie this hybrid sterility, as cytoplasmic male sterility is widely documented in other crops (Chen and Liu, 2014). Test backcrosses involving $F_1$ hybrids and their parents from populations in which the cultivated genotype was female support a cultivated cytoplasmic factor interacting with wild nuclear factor, as above. Interestingly, complete infertility of $F_1$ individuals was also observed when the reciprocal wild × cultivated

crossing was performed and backcrossing to either parent yielded viable progeny, suggesting the possibility of reciprocal cytoplasmic male sterility. However, the observation of both paternal and maternal plastidal inheritance in genus *Cicer* (Kumari et al., 2011) underscores the need for molecular data to track the origin of cytoplasmic factors (both mitochondria and plastids) to unequivocally test the possibility of cytoplasmic male sterility.

Finally, there is also cultigen-engendered variability for compatibility with *C. echinospermum*. This has been long known, including from the original identification of *C. echinospermum* as a distinct species (Ladizinsky and Adler, 1976; Pundir and Mengesha, 1995). In our study, we see such variation as well, in crosses with *C. echinospermum* from the Karabahce site (Table 1) that yielded self-fertile $F_1$–derived $F_2$s from some cultivars but not others. The self (in)fertility in $F_1$ interspecific hybrids in crosses with *C. echinospermum* from Karabahce does not partition by seed type, with self-fertile $F_2$s being produced with the 'desi'-type genotype ICCV 96029 and the 'kabuli'-type genotype Gokce, but not with the 'desi' genotype CDC Consul nor with the 'kabuli' genotype CDC Leader. This suggests factor(s) from the cultigen, rather than from *C. echinospermum*, contributing to underlying compatibility. That both Canadian cultivars failed to yield self-fertile $F_2$s suggests inadvertent coselection for (in)compatibility within the Canadian breeding program during cultivar development for the high-latitude chickpea production environment of southern Canada and the northern United States. It will be interesting to see whether these incompatibility factor(s) colocalize in the proximity of genomic regions containing genetic factors under selection during breeding, such as flowering time QTLs controlling responsiveness to photoperiod, or biotic constrains such as tolerance to the local Canadian strains of *Ascochyta rabeii* (Pass.) Labr.

Further work with the populations developed in the course of this study is necessary to elucidate the genes underlying these (in)fertility loci, to define the roles they may play in speciation or ecotypic divergence within natural populations of this crop wild relative, and to develop molecular markers for selection against sterility in marker-assisted introgression breeding. In particular, high-density linkage mapping with molecular markers from use of current approaches such as restriction site-associated DNA or skim sequencing could be particularly informative in determining how the loci we describe in our inheritance studies relate to chromosomal structural variation identified in earlier cytological studies (Ladizinsky and Adler, 1976; Ocampo et al., 1992; Tayyar et al., 1994; Galasso et al., 1996). Further characterization of cytoplasmic or cytoplasmic-genic male sterility factors that are suggested by our study may pave the way for the exploitation of heterosis (hybrid vigor) and use of hybrid technology in chickpea breeding. We conclude that, even in the absence of such information, our delineation of *C. echinospermum* into two distinct subgroups with hybrid sterility or hybrid breakdown informs and facilitates introgression breeding of traits uniquely present in this CWR from the secondary genepool of chickpea.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

## Supplemental Material Available

Supplemental material for this article is available online.


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