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Hybridization studies in *Vicia sativa* complex

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Abstract Some species in *Vicia sativa* complex, also called aggregate (C_{VS}), have economic and ecological advantages and they are frequently used for pasture, silage, and green manure. The main objective of this study was to explore the secondary gene pool for enhancement of common vetch (*Vicia sativa* L.) germplasm by genome introgressions via conventional crosses made between cultivated common vetch (*Vicia sativa* L. subspecies *sativa*) used as female parent with five other subspecies [*amphicarpa* (L.), Batt., *cordata* (Wulfen ex Hoppe) Asch. & Graebner, *macrocarpa* (Moris) Arcang., *nigra* (L.) Ehrh., and *segetalis* (Thuill.)], used as male parents. As indicated with very low seed sets, higher levels of sterility were the rule in hybrids in C_{VS}. Hybrids were confirmed using flower petal color and anthocyanin pigmentation markers. Chromosome count studies revealed that certain crossing-experiments were successfully produced interspecific hybrids. Certain offspring progenies in further generations were also obtained. Our studies clearly indicated that complex C_{VS} and hybrids obtained from these species consisted of 10, 11, 12, 13 and 14 pair of somatic chromosomes. Results indicated that there exist significant incompatibility alleles possibly expressing in the zygote or post-zygotic developmental stages within the C_{VS}. In conclusion, present study revealed that sufficient amount of seeds could be obtained from the crosses in the C_{VS} and this stimulates renewed interest in utilizing the secondary gene pool as a source of genetic variation in breeding programs of common vetch.

Keywords Breeding · Chromosome counting · Common vetch · Crosses · Pollen viability · Species aggregate

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Introduction

The genus *Vicia* L. is a member of the tribe *Fabeae* (also referred to as *Vicieae*/*Leguminosae*) of the subfamily *Papilionoideae* which includes *Vicia*, *Lathyrus*, *Lens* and *Pisum* (Hanelt and Mettin 1989). Pinning down exact numbers of species in genus *Vicia* L. is difficult due to cytological and morphological differences. Although numbers vary, it is estimated that this genus comprises about 166–210 annual or perennial species naturally grown in Europe, Asia, and North America, temperate regions of South America and tropical Africa (Hanelt and Mettin 1989; Tate and Ennenking 2006; El-Bok et al. 2015; Raveendar et al. 2015; Martin et al. 2018). Common vetch (*Vicia sativa*

L.), also known as tare, is widespread around many parts of world, including the Mediterranean basin, west and central Asia, China, eastern Asia, India, and the USA (Hueze et al. 2011). It is commonly grown as winter forage legumes for its high nutritional value, green manure, pasture, silage and hay. Since common vetch has the ability to grow in wider range of climate and soil conditions it is suitable intercrops for cereals to reduce diseases and help to improve soil properties (Acikgoz 1986; Seymour et al. 2002; Cakmakci et al. 2003, 2006; Chung et al. 2013; Mikic et al. 2014; Chai et al. 2017; Tiryaki et al. 2016; Dong et al. 2017; Geogieva 2018).

The Mediterranean region is accepted as the principal center of diversification, and the highest specific diversity of *Vicia* is found in Turkey and northwest Asia (Hanelt and Mettin 1989; Frediani et al. 2004; El-Bok et al. 2015; Raveendar et al. 2015; Dong et al. 2017; Martin et al. 2018). The number of polyploid species in the genus is very low, for instance among 87 species studied, ten were found polyploid ones, indicating the polyploidy is almost insignificant in the evolution of the genus *Vicia*, and the tribe *Vicieae* (Ladizinsky and Shefer 1982). Maxted (1993) divided the subgenus *Vicia* into nine sections using morphological key markers. These sections of subgenus *Vicia* are (1) *Atossa* (Alef.) Asch. & Graebner, (2) *Microcarinae* Maxted, (3) *Hypechusa* (Alef.) Asch. & Graebner, (4) *Peregrinae* Kupicha, (5) *Wiggersia* (Alef.) Maxted, (6) *Vicia*, (7) *Narbonensis* (Radzhi) Maxted, (8) *Bithynicae* (B. Fedtsch. ex Radzhi) Maxted and (9) *Faba* (Mill.) Ledeb. (Martin et al. 2018).

Existence of large structural differences in chromosome numbers and arrangements, great variation in the genome size along with the presence of allogamy, autogamy and even functional cleistogamy make classification more difficult in *Vicia* (Davis and Plitmann 1970; Hollings and Stace 1978; Zohary and Plitmann 1979; Raveendar et al. 2015). Phenological, morphological, cytological, karyological and cytogenetic studies were performed for taxonomic classification and genetic relationship studies. Extensive karyological studies based on chromosome size, centromeric index and banding patterns, and cytogenetic landmarks based on fluorescence in situ hybridization (FISH) or primed in situ DNA labelling (PRINS) have been established on several species (Navratilova et al. 2003). Biotechnological approaches using DNA and proteins have been used to identify divergence among wild populations in several vetch species (Haider and El-Shanshoury 2000; Shiran and Raina 2001; Arslan et al. 2012; Chung et al. 2013; El-Bok et al. 2014; Kim et al. 2015). Restriction fragment length polymorphism (RFLP), amplified fragment Length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), Start Codon Targeted (SCoT) markers, and microsatellites were used in *Vicia* species taxonomic classification. However, due to the much closer similarities resulted from inter- and intra-specific hybridization between certain accessions of different *Vicia* species rather than between accessions within each taxon were unable to assess the relationships between sativa species complex. Treating taxa within the *sativa* species complex whether species or subspecies seems to be a contentious issue for taxonomists of *Vicia* (Shiran and Raina 2001; Potokina et al. 1999, 2002; El-Bok et al. 2014; 2015; Raveendar et al. 2015; Chai et al. 2017; Han et al. 2017).

Within the genus *Vicia* several species are genetically, physiologically and morphologically related to certain species that are known as a *Vicia sativa* complex (C_{VS}) or sativa complex or sometime they are also referred as sativa aggregate. Members of the annual species of this complex are widespread in the Mediterranean countries and in the Near and Middle East (Hollings and Stace 1978; Zohary and Plitmann 1979; Ladizinsky and Shefer 1982; Hanelt and Mettin 1989; Gil and Cubero, 1993; van de Wouw et al. 2001, 2003; Schifino-Wittmann 2000; El-Shanshoury 2007). However, members of this complex are morphologically, karyologically and ecologically variable and were considered to be in active evolution (El-Bok et al. 2015) making identification difficult and confusing. The confused taxonomic status of the C_{VS} is probably due to hybridization between individuals of different cytotypes (Ladizinsky and Shefer 1982; Schifino-Wittmann 2000; Celiktas et al. 2006; El-Bok et al. 2015). van de Wouw et al. (2001, 2003) proposed six taxa of the sativa complex (C_{VS}) consisting of *amphicarpa*, *cordata*, *macrocarpa*, *nigra* (syn. = V. *angustifolia* L.), *sativa* and *segetalis* (Schifino-Wittmann 2000; El-Shanshoury 2007). Some members in C_{VS} were considered to be distinct species and were divided into several subspecies or varieties by several other researchers (Ladizinsky and Shefer 1982; Potokina 1997). Some species contain

more cytotypes within the same taxon. For instance, Schifino-Wittmann (2000) detected 2n = 12 and 2n = 14 plants of *nigra* in southern Brazil. Based on molecular data, Shiran and Raina (2001) revealed that seven species (*V. sativa* L., *V. incisa* Bieb., *V. nigra*, *V. cordata* Wulfen ex Hoppe, *V. amphicarpa* L., *V. macrocarpa* (Moris) Bertol and *V. angustifola* L.) within the C_{VS} shared a common ancestor and suggested that they could be referred as subspecies not species.

Earlier, some cytogenetic and karyological studies were conducted in C_{VS} (Donnelly and Clark 1962; Ladizinsky and Shefer 1982; Yamamoto 1968, 1974; Gil and Cubero 1993; El-Shanshoury 2007). Several experiments revealed that subspecies of C_{VS} consisted of 2n = 10, 12 and 14 chromosome complements. Within single or mixed populations of two, three or four subspecies, different chromosome numbers were observed in the natural habitats (Ladizinsky 1978; Ladizinsky and Temkin 1978; Zohary and Plitmann 1979; Han et al. 2017). A study of Ladizinsky and Shefer (1982) revealed that 2n = 10 cytotypes were found in secondary and artificial habitats while 2n = 12 were found in natural vegetation among dwarf shrubs, or in the maquis. On the other hands, the 2n = 14 cytotypes were found in dry habitats in steppes bordering the Mediterranean vegetation in Israel. Celiktas et al. (2006) reported that somatic chromosome counts of wild population of Vicia sativa L. were consistently 2n = 10, while the cultivar had 2n = 12 in Turkey. The same authors also noted that there were no significant differences between wild and cultivated cytotypes in plant height, seed number or pod number but the wild types had more branches, smaller seeds, and lower germination rates.

Numerous seed banks worldwide hold genetic resources of common vetch. Many common vetch varieties have been developed using the gene bank accessions. However, many unwanted characteristics have been co-transferred from other related gene pools. For instance, the presence of cyanoalanine, favism toxin, vicine and the neurotoxic peptide toxins in the seeds of vetch limits its usefulness in rations for certain animals and for human consumption (Enneking and Wink 2000; Firincioglu et al. 2007; Uzun et al. 2011; Kim et al. 2015). Furthermore, pod-shattering and diseases such as downy mildew (Abd El-Moneim 1993; Ahmed et al. 2000), or lack of winter hardiness (Acikgoz 1982a, b) restrict the use of common vetch accessions for improvement breeding studies. Interspecific hybrids obtained from interspecific crosses of different species such as within C_{VS} may provide several advantages. For instance, some forms of *amphicarpa* produce two types of pods: aerial and underground (subterraneous pods). These vetches have ability to survive in desert-like environments and produce herbage and pods (Acikgoz 1984; Ladizinsky 2014).

Increasing the observed genetic vulnerability in many crop species have stimulated renewed interest in utilizing the secondary and if possible tertiary gene pool as a source of genetic variation in plant breeding programs. Interspecific introgression to increase the genetic diversity is one of the solutions to the problem of genetic vulnerability and influences of founder effect. This study was undertaken to create interspecific hybrids between common vetch and 5 members within the complex C_{VS}. Genetic variations obtained could be used as a gene pool for obtaining or cloning resistance to various biotic and abiotic stress factors, insect and plant diseases, forage quality, earliness, etc., or transferring them to the cultivated lines (Celiktas et al. 2006; Tiryaki et al. 2016).

Materials and methods

Plant materials

Plant materials used in the present study consisted of six accessions of subspecies within the complex C_{VS}. Subspecies (subsp.) studied were *sativa* L., *amphicarpa* (L.), Batt., *cordata* (Wulfen ex Hoppe) Asch. & Graebner, *macrocarpa* (Moris) Arcang., *nigra* (L.) Ehrh., and *segetalis* (Thuill.). *Vicia sativa* L. subsp. *sativa* was represented with "W-1", (a Turkish line grown for hay and seed production), subsp. *cordata* was represented with "VIC701", (an accession originated from Egypt), subsp. *segetalis* was represented with "VIC420", (an accession from Greece), subsp. *amphicarpa* was represented with "VIC724", (an accession of Ukraine received from Leibniz-Institute of Plant Genetics and Crop Plant Research, Germany), subsp. *macrocarpa* was represented with "5283", (an accession from International Center for Agricultural Research in the Dry Areas, Syria, ICARDA), and subsp. *nigra* was represented with "K-1", (an accession from Turkey).

Hybridization studies (crosses)

All the *Vicia* accessions mentioned above were established and were grown in controlled greenhouse conditions using conventional vetch production practices (Acikgoz 1982b). Approximately 8–10 seeds per 30 L pots filled with a field soil were sown in four consecutive times with 15-day intervals in order to synchronize flowering of the parents. A total of 30 pots per accessions were made and after emergence of seedlings, one or two plants were left in each pot. In the crosses, a *sativa* line, K-1, was used as female parent and other five accessions (*amphicarpa, macrocarpa, segetalis, cordata* and *nigra*) were used as male parents. One-way crosses were made in order to detect the real hybrids based on anthocyanin and white petal flower color. Anthocyanin coloration of seedlings and purple flower color are known to be completely dominant to bright green seedlings and white flower color (Donnelly and Clark 1962; Chowdhury et al. 2004). Therefore, hybrid plants were easily detected by anthocyanin coloration at seedling stage and purple flowers during flowering period. Approximately 5–10 seeds from each F_1 cross were sown as mentioned above. Mature plants were selfed to obtain F_2 progeny of *sativa* x *amphicarpa, sativa* x *macrocarpa, sativa* x *segetalis, sativa* x *cordata* and *sativa* x *nigra*. Further studies were undertaken to obtain F_3 to F_7 generations.

Chromosome studies

Chromosome counts were obtained from cells of young root tips of hybrids and their parental lines according to reported protocols (Hollings and Stace 2006). About 10 seeds of each parent and five seeds from each hybrid were germinated on moist filtered paper in Petri dishes. Germination studies were initiated with seeds kept at 4 °C in a refrigerator for 1 week to break dormancy, and followed by incubation at room temperature at 18-24 °C for 2–3 days. During the germination, emerging roots were checked every day to catch root tips with accumulated cells at metaphase. Root tips of 1–2 cm were cut and transferred into clean tubes. These roots were treated with a saturated aqueous solution of α-mono-bromo-naphthalene and incubated at 4 °C for 8 h. Root tips were washed in running tap water on a steel sieve for 5 min and rinsed 3–5 times with tap water, then fixed with 99% glacial acetic acid (v/v) for 30 min. After fixation, root tips were washed twice with 70% ethanol (v/v) and stored at 4 °C in a refrigerator until use.

For staining, refrigerated root tips were rinsed 3 times with plenty of distilled water for 5 min and hydrolyzed at room temperature with 1 N HCl for 20 min followed by rinsing 3 times with plenty of distilled water for 5 min. Root tips were stained using 1% aceto-orcein solution for 2 h. Root tips were removed from the stain solution and incubated at room temperature in distilled water for 10 min. The meristematic areas of the root tips were excited into small pieces using a razor blade and then transferred on microscope slides and gently crushed between slides in a drop of 1% aceto-orcein (w/v) and mounted in 45% acetic acid (v/v). Suitable mitotic plates were observed with a binocular microscope, photographed under an optical microscope type Olympus with BX51/BX52 attached digital camera and stored in a personal computer. The chromosome counts of the subspecies and hybrids studied were confirmed in at least ten cells.

Pollen viability studies

Pollens were collected from parental lines and their hybrids for pollen viability studies. Pollen viability was assessed using the basic fuchsin staining (glycerin, gelatin and crystalline basic fuchsin). Pollen grains were transferred on a microscope slide and washed with 70% ethanol (v/v) on a slide to remove oily substances and the remaining of anther tissues (Trognitz 1991). Remains of alcohol was allowed to evaporate by heating the slide. A drop of melted glycerin jelly with basic fuchsin dissolved in a water bath at 50 °C was put on slide to embed the pollen grains. Embedded pollens were covered with a coverslip. Slides were incubated in a heater at 50 °C for 1–2 min and dried upside down before at least one-day storage. Light microscope type Olympus BX51/BX52 attached digital camera was used to assess the pollen viability. Viable pollen grains appear pink colored and well-rounded while nonviable pollen grains were not stained and looked shrunken. The percentage of viable and unviable pollens was measured by examining approximately 100 pollen grains in two different specimens.

Results and discussion

A total of six hybrids were obtained using a line belonging to subspecies *sativa* as female parent with the genetic marker of white flower color known governed by a single recessive gene. On the other hands, the subspecies used as male parents had purple flower which is completely dominant to white flower color. Observations revealed that purple flower color was associated with anthocyanin pigmentation in the stems of seedlings and mature plants while white-flowered plants showed none of this pigmentation. These morphological differences were used as selection and confirmation markers for confirmation of successful hybridizations between subspecies *sativa* and other accessions. With the use of anthocyanin coloration marker, we could identify successful hybrids immediately after seedling emerged by the visualization of reddish-purple color in the stems. We selected and utilized F_1 hybrids that contained reddish-purple color in the stems and purple petal colors. Since the presence of anthocyanin in the stems of seedlings and purple color in petal were dominant to light green shoots and white flower color, heterozygote (hybrids) contained these markers while those female *sativa* plants contained light green shoots and white flower color. Hybrids with heterozygote genotypes produced F_2 progenies with a 3:1 ratio of reddish-purple color in the stems with purple petal colors, and light green shoots with white flower color. For instance, when a total of 59 F_2 plants obtained from selfed *sativa* x *nigra* hybrids were studied, a total of 59 F_2 plants consisting of 43 purple and 16 white flower were identified. This segregation was typically a 3:1 segregation and confirmed previous reports that the color of the flower was governed by a single gene (Donnelly 1958; Donnelly and Clark 1962; Chowdhury et al. 2004; Ladizinsky 2014).

Chromosome counts

Chromosome counts were performed in all parents and their hybrids using at least 3 root tips obtained from approximately 10 seeds of each parent and five seeds from each hybrid. For each plant sample at least 10 counts were performed. *Vicia sativa* subsp. *sativa* used in all crosses as female parent, contained 2n = 12 chromosomes. Subspecies *cordata* and *segetalis* had 2n = 10 chromosomes, *macrocarpa* and *nigra* had 2n = 12 chromosomes while *amphicarpa* had 2n = 14 chromosomes in root tip cells. Chromosome counts in all the F_1 hybrids obtained in the hybridization studies revealed that the number of chromosomes was an average of the two parents (Fig. 1). Mitotic chromosome number (2n) of hybrids ranged from 11 to 14. Mitotic chromosome number of hybrid *sativa* x *macrocarpa, sativa* x *cordata, sativa* x *segetalis, sativa* x *amphicarpa* and *sativa* x *nigra* were found 2n = 12, 2n = 11, 2n = 11, 2n = 13 and 2n = 12, respectively (Fig. 1). Observed somatic chromosome numbers of 10, 11, 12, 13 and 14 within the parental lines and their hybrids indicated that there were three different basic chromosome numbers x = 5, 6 and 7. This indicated that the basic chromosome number was n = 7, from which n = 6 and n = 5 have originated in *Vicia*. In close agreements with our studies, the same somatic chromosome numbers of *Vicia sativa* subspecies reported in earlier studies were also counted in the present study (Yamamoto 1968; Hollings and Stace 1974; Ladizinsky 1978; Ladizinsky and Temkin 1978; Zohary and Plitmann 1979).

Analyses showed that parental accessions were diploid, and no aneuploidy and higher ploidy levels were detected in the present study. Results revealed that the shape and length of somatic chromosomes of the complex C_{VS} were different. We noted that there seemed an inverse relationship between chromosome number and the size of chromosome within the complex C_{VS} (Fig. 1). For instance, there were a reduction in chromosome size per se 2n = 14 > 2n = 12 > 2n = 10 for the complex C_{VS}. The differences in number, shape and length of chromosomes indicated the complex C_{VS} have great cytogenetic property that could be an indication of genetic diversity (Potokina et al. 2002; Chung et al. 2013; Kim et al. 2015). Although it is not universally acceptable in plant genomes, it could be stated that the greater genetic distance between parents, the greater the amount of genetic recombination and hybrid vigor could be obtained (Ince et al. 2010; Karaca and Ince 2018a). Higher cytological differences may cause high degree of segmental homology between the parental genomes that may result in forming a segmental alloploid, characterized in meiosis by multivalent associations, producing unbalanced gametes (Ladizinsky and Shefer 1982).

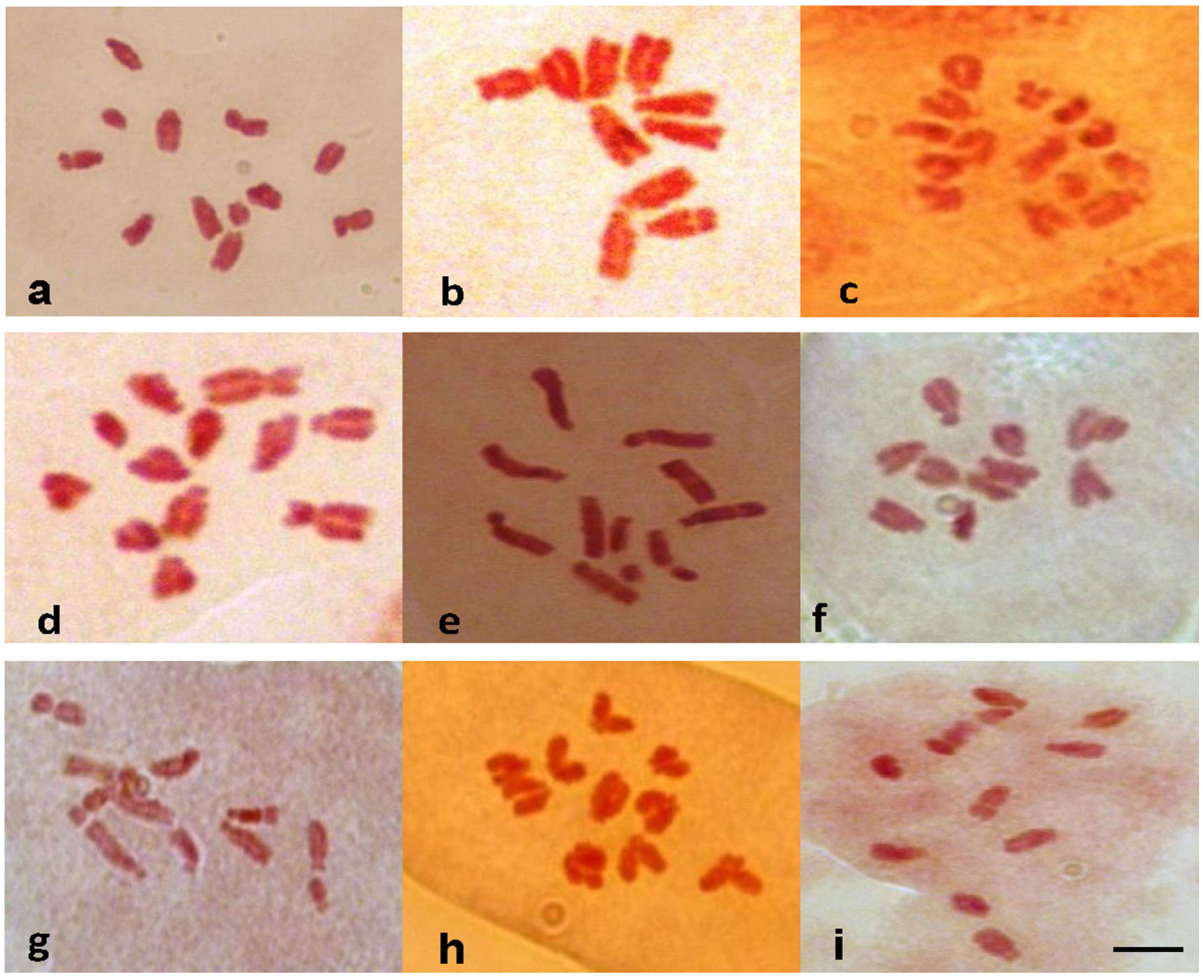

Fig. 1 Somatic chromosomes at mitotic metaphase of **a** *sativa* 2n = 12, **b** *nigra* 2n = 12, **c** *amphicarpa* 2n = 14, **d** *macrocarpa* 2n = 12, **e** *cordata* 2n = 10, **f** *sativa* × *cordata* 2n = 11, **g** *sativa* × *macrocarpa* 2n = 12, **h** *sativa* × *nigra* 2n = 12, **i** *sativa* × *amphicarpa* 2n = 13, (Scale bar = 5 μm)

Pollen viability

Viable and dead pollens were visualized (Fig. 2) and the viability rates of parents and their hybrids were determined. Pollen viability of the parental lines and accessions ranged from 100% in subspecies *macrocarpa* and *amphicarpa* to 44% in *nigra*. On the other hands, pollen viability of the hybrids ranged from 8.1% in *sativa* x *segetalis* hybrid to 0.5% in *sativa* x *nigra* hybrid. In general, pollen viability of lines and accessions was found to be very high in all of the parents with the exception in subspecies *nigra*. Pollen viability of subspecies *sativa* was 98.1%, *macrocarpa* 100%, *segetalis* 99%, *nigra* 44%, *cordata* 91%, and *amphicarpa* 100%. Analysis revealed that *sativa* x *segetalis* (8.1%), *sativa* x *cordata* (7.2%) and *sativa* x *amphicarpa* (7.1%) produced better pollen viability than *sativa* x *nigra* (0.5%) and *sativa* x *macrocarpa* (3.1%). Based on the study of more than 500 pollen grains, we concluded that when pollen viability rates of parental lines were high, hybrids had higher pollen fertility, thus; higher seed sets were obtained. Although this study does not have any genotypic analysis, using accession data we could state that viability and seed set of interspecific crosses can depend on the genotype of the lines used as parents. Low level of pollen viability in hybrids associated with the low level of seed retention. Zohary and Plitmann (1979) observed just negligible percentage of normally stained pollen in F_1 plants of *sativa* x

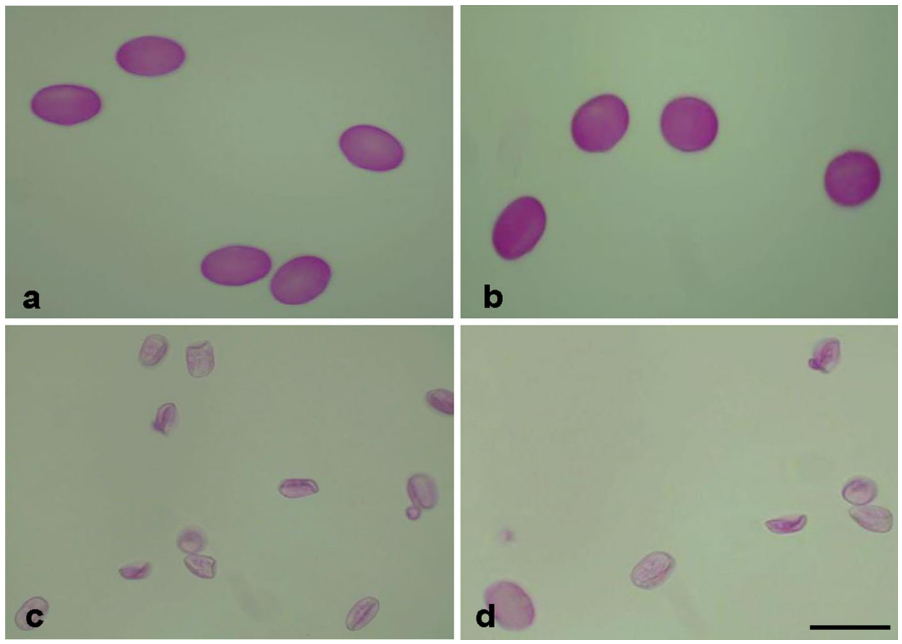

Fig. 2 Representation of fertile pollens in the parents (**a**, **b**), dead pollens in F_1 hybrids (**c**, **d**) (Scale bar = 40 μm)

cordata hybrids. Watanabe and Yamada (1958) also reported very low pollen fertility in *sativa* x *angustifolia* hybrids. In the present study, it was observed that high rate of mortality in the pollens could be correlated a low seed set of 1 or 2 seeds per pod in *sativa* x *nigra* and in *sativa* x *macrocarpa* hybrids. Earlier, Cooper (1958) reported that intra-specific crosses in *Vicia sativa* were quite fertile, but they could not obtain seeds from 24 hybrid *Vicia* species. Watanabe and Yamada (1958) reported that *sativa* x *angustifolia* hybrids had very low (5%) pollen fertility. Also Cubero (1982) reported that hybridizations between subspecies *sativa* and *macrocarpa*, *amphicarpa* or *angustifolia* were not successful, there were very low seed sets, poor pollen fertility or no seed germination.

Varying rates of viability and mortality among the hybrids obtained by crosses of 6 subspecies clearly showed that some subspecies could hybridize more easily than others. We speculated that this was probably due to the existence of incompatibility alleles in some subspecies than other (Abdalla 1977). In the present study, we did not use reciprocal crosses because of the selection markers were valuable only crosses in which female parent was *sativa*. High rate of mortality in the crosses were probably due to incompatibility, which causes poor agronomic qualities of the progeny, distorted segregation or sterility and limited recombination. The low level of pollen viability observed in certain hybrids directly correlated with their infertility. All living hybrids produced mostly single-seeded, rarely two-seeded pods (Fig. 3). For instance, hybrids between *sativa* x *segetalis* produced a total of 60 pods, 58 of which consisted of a single seed while two had two seeds. A total of 17 pods consisting of 16 single seeds and one with two-seeds were obtained from hybridization of *sativa* x *cordata*. Similarly, 16 pods and 16 seeds in *sativa* x *amphicarpa* hybrid, 17 pods and 18 seeds in *sativa* x *nigra* hybrid, 34 pods and 42 seeds in *sativa* x *macrocarpa* hybrid were obtained. We speculated that the differences in chromosome numbers or chromosome structures probably prevented regular matching in meiosis. This infertility in F_1 hybrid plants is consistent with the literature. Many researchers (Donnelly and Clark 1962; Mettin and Hanelt 1964; Zohary and Plitmann 1979; Ladizinsky 1981) reported that pollen viability and seed set were very low in crosses between *Vicia* species and subspecies at F_1 generation. We speculated that crosses between *sativa* × *segetalis*, *sativa* × *cordata* and *sativa* × *amphicarpa* produce homomorphic bivalents that support better survivorship and fertility (Ladizinsky and Shefer 1982).

Fig. 3 Single-seeded pod examples in F_1 hybrids. **a** *sativa* × *segetalis*, **b** *sativa* × *cordata*, **c** *sativa* × *amphicarpa*

State of interspecific crosses in generations

Crossing studies at the interspecific level within the complex C_{VS} were difficult, and, therefore, limited number of hybrid seeds were obtained in our experiments. It was speculated that difficulties might have come from segregation distortion, suppression of recombination, and linkage drag since they are often encountered in interspecific crosses (Watanabe and Yamada 1958; Rousi 1961). As indicated with very low seed sets, higher levels of sterility were the rule in hybrids between subspecies *sativa* x other subspecies of the complex C_{VS}. It was interesting to note that even when some F_1 hybrids showed considerable hybrid vigor as observed from plant heights and pod sizes; members of F_2 generations were mixtures of etiolated leaves (also some variegated), lethal and some normal types. Watanabe and Yamada (1958) reported crosses between subspecies *sativa* and subspecies *angustifolia*, using *angustifolia* as a male parent. These authors found that pods of hybrids resembled the pods of subspecies *angustifolia.* In further studies, the authors showed that chromosome pairing at meiosis was very poor, and as a consequence pollen fertility was low. In our study, it was observed that after the F_2 generations, fertility of some hybrids started to improve and hybrids held good seed set especially after the F_3 generation (Donnelly and Clark 1962; Ladizinsky 1981). Researchers attributed this improvement as fixation of chromosome numbers in advanced generations. Zohary and Plitmann (1979) reported that many crosses between subspecies with *sativa* did not produce viable hybrids. These researchers found that the rate of seed retention in the F_1 of the *sativa* x *cordata* hybrids was only 12–16%, whereas it was 90–95% in the parents. They also reported that fertility has improved after the F_2 generation. In the present study, improvement of viability and fertility in F_3 and F_4 generations of some crosses were probably due to the fact that some gametes were eliminated during zygote formation and euploid genomes become advantages in further generations.

Seeds from parental and hybrids were harvested and 20 seeds from each sample were sown to obtain F_1 plants. Majority of seed either not germinated, or germinated but did not survive, or did not produce seeds. A total of 27 seeds from *sativa* x *amphicarpa,* 61 seeds from *sativa* x *macrocarpa*×20 seeds from *sativa* × *segetalis*, 27 seeds from *sativa* x *cordata* and 59 seeds from *sativa* x *nigra* were harvested. Varying

number of F_2 plants were grown but the highest infertilities were observed in F_2 generation. However, fertility rates increased during F_3 and F_4 generations. A total of 81 lines consisting of subspecies 2 *sativa* × *amphicarpa,* 46 *sativa* × *macrocarpa,* 9 *sativa* × *segetalis,* 11 *sativa* × *cordata* and 13 *sativa* x *nigra* were developed. Further studies using reciprocal crosses within the complex C_{VS} would produce additional information regarding elimination, recovery and fertilization.

In order to improve the rate of viability and fertility of hybrids obtained from interspecific crosses within the complex C_{VS}, suitable lighting, temperature and other environmental conditions, hormonal treatments, embryo rescue, embryo culture, decapitating styles and grafting the floral parts should be considered (Karaca and Ince 2018b; Karere et al. 2010). However, when the incompatibility is not in the stigma and/or style affects the zygote or post-zygotic development, these techniques would not provide amendments on the fertility ratio. Alternative approaches such as chromosome grafting based translocations via irradiating fragments of donor chromosomes to the recipient genome can be considered (Karere et al. 2010).

Conclusions

The genus *Vicia* has long been an important subject of scientific research because it contains several species of economic importance and provides interesting features for ecological and genetic aspects for plant scientists and breeders. In the present study, we reported several interesting findings that could be used in the breeding and genetic studies of *Vicia* species. We concluded that successful interspecific hybridizations within the complex C_{VS} were possible but hybridizations have some bottlenecks. The highest infertilities were observed at F_1 and F_2 generation while fertility rates increased as generation progress forward and full fertility were obtained during F_3 and F_4 generations. These findings indicated that interspecific crosses within the complex C_{VS} could be used to manipulate genetic and epigenetic traits involving in processes of hard seed, winter hardiness, and resistance to bacterial and fungal diseases, root knot nematodes, spider mites and aphids. However, higher success in interspecific hybridization within the complex C_{VS} is still difficult, novel approaches such as bridge crosses, mentor pollen application, stigma grafting and reverse breeding approaches would increase the rate of success within the hybridization studies within the complex C_{VS}. Upon obtaining successful genome transfer (hybridization), interspecific hybrids as genetic stocks could be used to introduce some nuclear and/or cytoplasmic genetic traits with desired economic values to breeder lines. Therefore, introgression via interspecific crosses in common vetch not only definitely enhance breeding efforts but also expand wealth of genetic resources.

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