Assessment of level and differences among *Vicia faba* L. genotypes for their degree of cross-fertilization and for their paternal mating success, based on a polycross field experiment.

MSc Thesis

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Abbreviations

ANOVA	-	Analysis of Variance
CMS	-	Cytoplasmic Male Sterility
DNA	-	Deoxyribonucleic acid
DOC	-	Degree of cross-fertilization
FAO	-	Food and Agriculture Organization
ICUPA	-	International Union of Pure and Applied Chemistry
NP	-	Neighborhood proximity
KASP	-	Kompetetive Allele Specific PCR / K-Bioscience Allele - Specific PCR
PCR	-	Polymerase chain reaction
PMS	-	Paternal Mating Success
SNP	-	Single nucleotide polymorphism

1. Introduction

1.1 Relevance of *Vicia faba* L

Faba bean (*Vicia faba* L) is an important food legume all over the world since it is useful for both human consumption and animal feeding and can be adaptable to many regions and. It is one of the earliest domesticated Old World agricultural crops, and its origin is probably in a site in North-West Syria (Tanno and Willcox, 2006). Faba bean production is more evenly distributed around the world than most other grain legumes (Jensen et al., 2010), yet it is not grown in very large amounts in the tropics. The major producing countries were China with 1.42 million metric tons (mmt), Ethiopia with 0.84 mmt and Australia with 0.33 mmt. In 2014 China contributed 34% of the global production area of faba bean, followed by Ethiopia, Morocco and Australia with 22%, 9% and 8% respectively (FAOSTAT 2014).

The diversity of faba bean is distinguished into two main groups, the small seeded and large seeded type. Faba bean is a significant source of protein in developing countries and use for human food and animal feed in industrialized countries (Duke, 1981). In Tunisia, small seeded and large seeded faba beans are used for both purposes (Mohamed and Halima, 2011). In China, which is largest producer and consumer of faba bean, the crop is mostly used as vegetable and as such it is one of the important food in Chinese diet. Some Asian countries such as Thailand, Myanmar and Vietnam also have diverse utilization of faba bean with some extent. In developed countries, faba bean is gradually being considered as a protein source for human consumption because of the changes in eating pattern. As an example, it could be an alternative nutrient source for vegetarians and vegans.

In general, faba bean is a highly profitable grain legume, especially if the economic benefits of biologically fixed nitrogen and enhanced weed and disease control in subsequent crops are considered (Preissel et al., 2015). It is also beneficial in legume–cereal rotations in cropping systems, where it is used as a break crop for cereals like wheat, barley and maize (Amanuel et al., 2000; Lopez-Bellido et al., 2006).

Faba bean is a partially allogamous grain legume with 2n= 2x= 12 chromosomes (Satovic et al., 2013). The reproductive system of faba bean is both self and cross-fertilization. Cross fertilization is mediated by bee pollinators who transfer the pollen grains between different flowers and furthermore cause a mechanical stimulation of the flower called tripping. Self-fertilization also needs tripping. However, some flowers can be self-fertilized without

tripping and this is considered as autofertility (Link, 1990). The autofertility rate in faba bean varies from 1 to 98%(Link, 1990) whereas the degree of cross-fertilization is between 10 to 70% (Bond 1987; Salih et al., 1994). Higher degree of cross-fertilization is typically connected with lower autofertility (Link, 1990).

However, the yield of faba bean is relatively unstable in comparison with many other major crops, which is thought to be one important reason for the low inclusion rate of leguminous crops in European agriculture in particular (Cernay et al., 2015). Optimal yield production of faba bean also depends on symbiosis with *Rhizobium leguminosarum* to produce nitrogen-fixing root nodules as well as on the pollination services of wild and domesticated bee populations to ensure both optimal seed set and outcrossing rates. Pollinator insufficiency could lead to 64% yield loss (Nayak et al., 2015).

To fulfill the demand for food and feed protein sources is another challenge apart from yield stability. In order to maximize the nutritive value of faba bean, it is important to minimize anti-nutritional factors such as vicine. Vicine and convicine content in faba bean are naturally high (Khamassi et al., 2013) and have been shown to lower protein digestibility and energy content in a variety of animal feeding studies. Moreover, these pyrimidine glucosides cause a serious and potentially fatal condition known as favism in genetically predisposed humans (Ray and Georges, 2010). Apart from the yield instability and quality, biotic and abiotic stresses are other traditional constraints in faba bean cultivation (Cubero, 2012). The mentioned constraints are addressed by faba bean breeders in order to improve the performance of the crop.

1.2 Heterosis in faba bean breeding

The major constraints for faba bean production are insufficient yield and yield stability. To improve these traits, one approach in faba bean breeding is a better exploitation of heterosis. Heterosis is a superior feature for yield and yield components mainly caused by the genetic combination of different genes in one genotype (heterozygosity). Heterosis is generally defined as difference in performance of an F_1 hybrid when compared with the mid-parent performance (presumed the parents were unrelated and homozygous) (Becker, 2011; Zeid et al., 2004). This is true not only for the partially allogamous crops but also for some autogamous crops like green gram (Narasimhulu et al., 2016). In faba bean, heterosis for grain yield bean where F_1 hybrids more than both better parent and mid-parent means has been reported (Zeid et al., 2004). The exploitation of heterosis present in faba bean provides

a good chance to increase and stabilize their performance (Ebmeyer, 1988). However, the degree of cross-fertilization can influence the exploitation of heterosis (Stelling et al., 1994). A higher degree of cross fertilization is leading to higher heterozygosity and hence to a higher amount of heterosis and that kind of genotypes might have a higher yield stability. The level of heterosis for yield and the degree of cross-fertilization are important for breeding of populations such as synthetic populations and for hybrid breeding (Link et al., 1994a).

In faba bean, the choice of the suitable breeding category is difficult, because its natural reproductive system is partial allogamy. Hybrid breeding allows maximum exploitation of heterosis and this could be a solution to reduce the problem of yield instability in faba bean (Stelling et al., 1994; Zeid et al., 2004). To perform hybrid breeding, a cytoplasmic male sterility (CMS) system is the most promising hybridizing mechanism. However commercial hybrid production of faba bean is still not a reality although two new CMS systems and effective restorers have been discovered (Link et al., 1997). These systems are not giving a stable male sterility since pollen sterility is very low.

An alternative way to achieve higher heterosis is producing synthetic cultivars (Suso, 2005). Synthetics express a part of the heterosis because they are only partly inbred (Ghaouti and Link, 2009). In faba bean breeding, synthetic varieties were repeatedly recommended over line varieties to increase the yield and yield stability of faba beans (Ebmeyer, 1988; Link et al., 1994a; Stelling et al., 1994). Population varieties such as synthetics have the possible chance to get high heterosis and the advantageous effects of heterogeneity especially on yield stability (Stelling et al., 1994). Heterozygous genotypes are less susceptible to environmental influences than homozygous ones, and that heterogeneous populations are better buffered against such stresses than homogeneous ones (Becker and Leon, 1988). In general, the extent of genotype x environment (GxE) interactions as determined by the adaptation of genotypes across geographical areas and cropping years (Kang, 1998; Annicchiarico, 2002). Significant (GxE) interactions of faba bean varieties and breeding lines for yield were reported by Stelling et al., (1994), Kittlitz et al., (1993) and Link et al., (1996). Therefore, hybrid breeding and synthetic breeding are major faba bean breeding categories for yield and yield stability now a day (Gnanasambandam et al., 2012). Development of new plant molecular biology could assist to accelerate the faba bean breeding.

1.3 SNPs in faba bean breeding

Due to the potential of using molecular tools for faba bean cultivar development, the use of molecular markers has improved significantly for many breeding purposes and some Single Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) markers have been developed (Oliveira, 2016). SNP markers have become extremely popular in plant molecular genetics due to their genome-wide abundance (Mammadov et al., 2012). Single nucleotide polymorphism (SNP) marker analysis is based on single base change in a DNA sequence, with an alternative of two possible nucleotides at a specific position. A very small number of SNPs have been discovered prior to 2014 (O'Sullivan and Angra, 2016). A SNP occurs when a single nucleotide (A, T, C or G) in a specific position in the genome differs between members of a species or between paired (homologous) chromosomes in an individual. SNP marker analysis can be used to analyze genetic diversity, create genetic maps, and utilize marker-assisted selection breeding in many crop species (Hannah et al., 2015). Genetic mapping for faba bean using SNP are being developed because of they are cost effective (Khazaei et al., 2014).

There are many platforms to use SNP for crop improvement (Segmen et al., 2014). In this research, we employed a new allele-specific SNP based on a PCR genotyping system developed at KBiosciences. The Kompetitive Allele Specific PCR (KASP) is based on competitive allele specific polymerase chain reaction (PCR) and it is suitable for SNPs and insertions and deletions (Indels). Many of the polymorphic intron sequences were converted from cleaved amplification polymorphic sequence (CAPS) to Kompetitive Allele Specific PCR (KASP) format (Cottage et al., 2012). More recently, a SNP-based consensus genetic map was constructed by Webb et al., (2016) and the authors reported design of individual KASP assays for 845 SNPs.

1.4 Aim of the work

Knowing the degree of cross-fertilization is crucial for breeding a synthetic variety, since this information is needed to predict the heterotic part of a synthetic's performance (Becker, 1988). Moreover, it may be important to know the contribution of father plants to the total of the cross-fertilization. Paternal mating success, the contribution of father genotypes to the successfully cross-fertilized seeds, is also important and can have an influence on the genetic make-up of the synthetic (Tacke, 2017). The distinct roles of the father "pollen donor" and the mother "pollen acceptor" might be due to the fitness or of the quantity of

the pollen (Gasim and Link, 2009). Therefore, the contribution of mother genotypes for degree of cross-fertilization and mutual paternal mating success of father genotypes will be taken into consideration. Additionally, the different levels of genotypes effects on degree of cross-fertilization and paternal mating success will be assessed by using F_1s and inbred lines. It is assumed that plants which grow in close proximity to each other will probably have a higher chance of cross-fertilization between each other than being cross fertilized by more distant plants. This assumption will be tested by modelling the extent of neighborhood proximity and by calculating the chance of a plant being father "pollen donor". In this research, the question will be addressed whether the neighborhood proximity between a mother and father plant can influence the paternal mating success.

2. Materials and Methods

This study was conducted at the Division of Plant Breeding, Georg-August-Universität, Göttingen. The cultivation period was March to September of 2015. The plant materials used in this work were derived from the Göttingen Winter Bean Population (GWP) which was founded in 1989. It was decided to use the mixture of lines and F_1 genotypes for a so called polycross to compare the degree of cross-fertilization based on different inbreeding levels. According to Link 1990, autofertility and cross-fertilization are negatively correlated in faba bean, the six different genotypes which were selected for this experiment have diverse autofertility rate and the autofertility rate for two F_1 genotypes are not yet known.

In this experiment, a "complete neighbor balance" design (Morgan, 1987) was used for sowing the polycross with two factors, mother plant or pollen acceptor and father plant or pollen distributor for polycross (Brünjes, 2014). Hence, by the consequence of using this "complete neighbor balance", each genotype possesses every other genotype as direct neighbor by the same number of cases (Morgan, 1987) and every genotype basically should have the same chance to cross-fertilize with every other genotype. Eight genotypes for this polycross with eight blocks and a contribution of eight individual plants per genotypes in each of the eight blocks are included. Although these eight genotypes have different autofertility levels, in the selection process it was also emphasized to get almost the same flowering dates for each of them. Color labels clipping to individual plants to mark the nodes which had the highest chance of cross-fertilization and to notice the pods from marked nodes was conducted while the genotypes were standing in polycross by using different color labels and clipped at different date.

After harvesting, the seeds from eight genotypes were sown for SNP marker analysis to distinguish whether these seeds (those offspring genotypes) were self or cross-fertilized and to identify their corresponding fathers. The degree of cross-fertilization and paternal mating success of these eight genotypes were calculated based on SNP analyzed results. Cross-fertilization is represented by the proportion of the cross-fertilized seeds of a single mother plant. Paternal mating success is calculated by the frequency of cross-fertilized seeds of specific father genotype with one particular mother genotype upon the total number of crossed seeds by this one particular mother. The proximity of neighbor plants influences the degree of cross-fertilization and the mutual paternal mating success of

genotypes was an assumption (hypothesis) in this experiment.

2.1 Plant Materials

To analyze the degree of cross-fertilization and paternal mating success, eight different genotypes with the contribution of six different lines and two F_1s were selected to be grown in a polycross (Table 1). These genotypes were selected base on following morphological characters, which are crucial for studying cross-fertilization. Autofertility level and begin of flowering dates of the inbred lines were considered to get an equal chance of cross-fertilization among the genotypes. In additions, disease resistance level, lodging and good surviving after winter were included as criteria. Entry numbers were assigned to represent every different genotype from 1 to 8.

Table 1. *Vicia faba* **genotyes used for the ploycross**

Entry numbers	Pedigree names of eight genotypes
1	S_85
2	S_46
3	S_235
4	F_1 (S_19 x S_35)
5	F_1 (S_25 x S_217)
6	WAB_EP02_Fam157
7	S_145
8	S_199

2.2 Experimental design

"Complete neighbor balance" (Morgan 1987) design was arranged as polycross for the eight genotypes. According to Morgan (1987), frequency of being neighbor by every each other genotypes were equal when considering the entire polycross.

Every plant of the eight genotypes acted simultaneously as female and male parent hence were assigned as mother and father factors and blocks were also considered as an experimental factor. There were eight replications (individual plants) per block and per genotype and a total of eight blocks (replicates). The plant spacing was 22 cm within rows, 40 cm between double rows with a distance of 22 cm between single rows.

Figure 1 Experimental design of polycross. The first plant of genotype one in block one was marked as single plant no.1 and block one includes single plant number 1 to 64. Eight replications of every genotype are included in each block. Therefore, the number of individual plants per genotypes per block is eight and eight genotypes contribute a total of sixty-four plants to the polycross.

2.3 Yellow and blue label clipping

It is very important to make sure that every single plant of each genotype is able to contribute its pollens to the cross-fertilization. In this case, it was quite difficult to catch 100% flower synchronization date, eight different genotypes were used so far. Therefore, label clipping of the plants in the polycross was conducted at two different times with different label colors. This action is important not only to identify the pods which had the highest chance of cross-fertilization but also to classify the lower chance pods.

Yellow labels were clipped to notice that at least three flowers of the first inflorescence of each single plants already started flowering. This clipping time was done by scoring according to the BBCH (ANNEX1) scale to access flowering differences between genotypes. Usually, yellow and blue labels were clipped at least at two nodes distance between each other. Blue label clipping is necessary to mark which inflorescence has the highest chance to cross with every other genotype. Therefore, blue labels were clipped at the date of almost all plants having highest flowering rate and almost synchronized flowering. The labels were fitted just above the node and inflorescence which presented a freshly opening inflorescence on that date (Figure 2).

Figure 2. Blue and yellow labels on the faba bean plants during the flowering time and when the pods are mature. In this figure, the position of color labels on the plant immediately after clipping at the flowering and when the pods matured are compared to visualize.

The blue clipped node is marked for "0, above and below adjacent ones are marked for "1" and "-1" respectively and kept on marking for the rest nodes and the same procedure for another tiller (Figure 3).
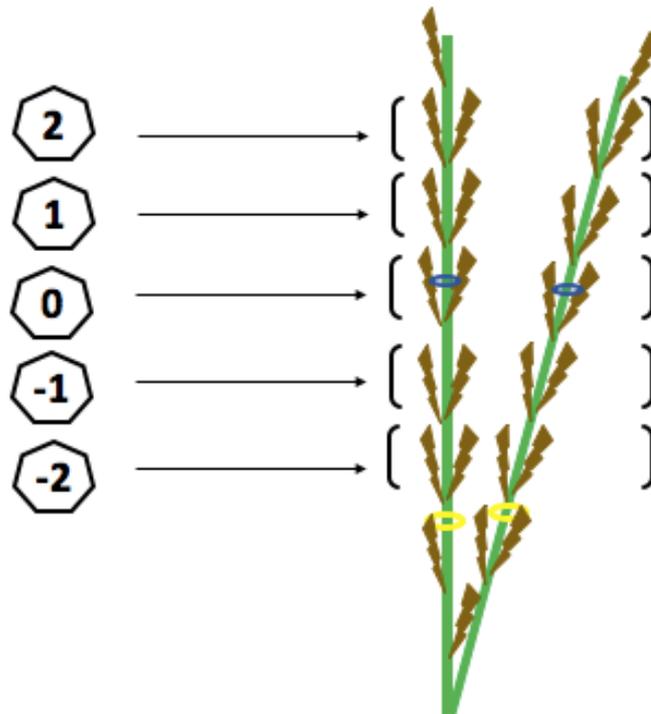
Figure 3. Marking nodes number by blue label. The node immediately below the blue label is node "0", above the node "0" is node "1" and below is node "-1".

2.4 Harvesting and sowing the offspring seeds from polycross

2.4.1 Harvesting

Harvesting of the polycross started when all single plants and pods were matured (September 2015). Harvesting was done manually and separately for each single plant. Intensive and detailed harvesting was done by keeping the pods separately according to the nodes position to provide the pod distribution and detail information across the tillers of each single plants. In this case, small white bags were used to keep harvested pods separately based on node position and brown bags to collect all white bags as a single plant harvest. The node position was written down on the white bags and the full information of single plant was on the label of the brown bag (Figure 4).

During harvesting, number of tillers, number of nodes deviation from yellow and blue label, number of pods per node, number of pods per tiller and total number of pods per plant were collected. The main stem was determined as tiller one and the first tiller which is coming from the main stem was named tiller two. Axillary shoots were discarded. If the pods from node "0", "1" and "-1" had vigorous and nice pods, then the remnant pods were bulked (top versus bottom of the such tiller). Fully attention was given on the number of pods from each node until at least 12 seeds per single plant was achieved, needed to conduct the SNP analysis (paternity test).
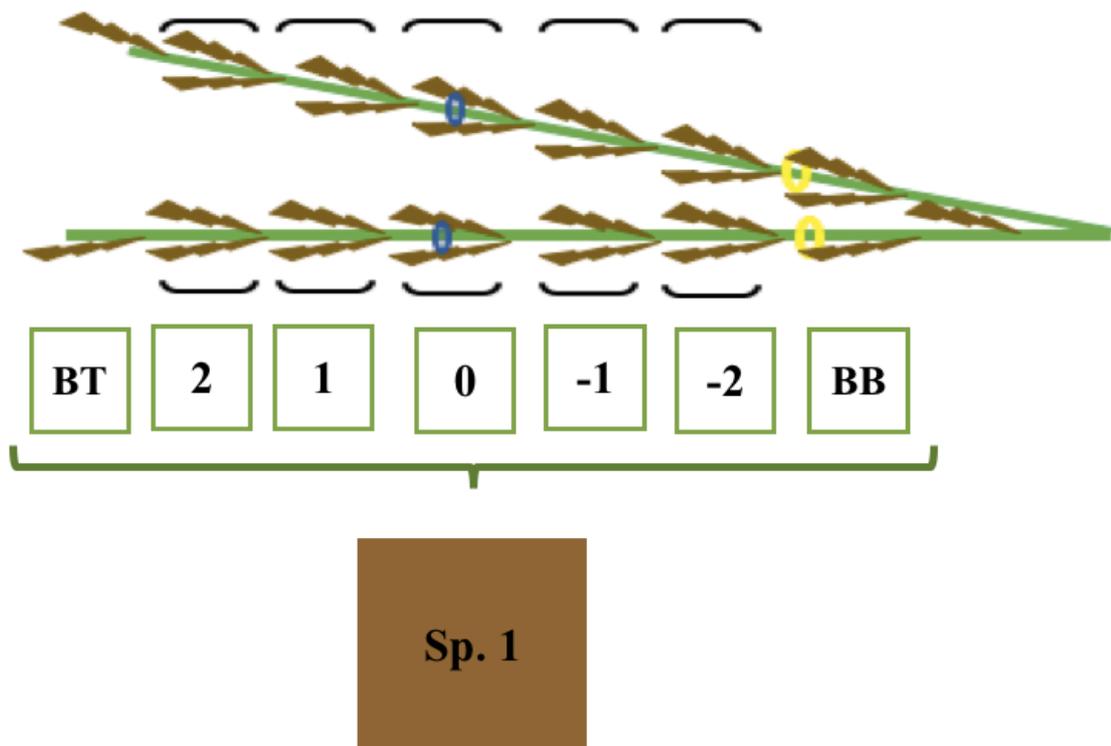
Figure 4. Harvesting procedure of each single plant. The pods from different nodes were individually put to a small bag and all of the small bags were collected into a large bag with the full label of a single plant (SP). When node 0, 1 and -1 had enough pods (at least four pods) the other were bulked as top(BT) and bottom (BB) respectively. The corresponding SP number will be on that bag with full label which is describing the all information of that plant.

2.4.2 Sowing the next generation of polycross

After harvesting the polycross, a number of selected seeds was sown to conduct a SNP analysis by which distinguish whether those seeds were generated by self-fertilization or cross-fertilization.

Ideally, it was attempted to plant twelve seeds per mother single plant. The number of seeds sown could vary depending on actually harvested number of pods per node. It was supposed to use the pods from node "0" as first priority, and followed by "1" and "-1" pods until a sufficient number of seeds were sown. In some cases, the numbers of seeds sown was slightly higher than twelve. The deviance from node "0" should be as low as possible. Since the node "0" was marked at a flower concurrence date at the begin of the flowering period, the positive node numbers had higher chance of cross pollination than negative ones since the node "0" was marked at the highest flower synchronization date for all genotypes, positive node numbers were preferred over the negative node if seeds were not enough by those 3 nodes. It means, "1" was preferred over "-1", "2" over "-2" and so on. At sowing

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those seeds, detail information was recorded such as number of seeds per pod, number of pods per node sown and total number of seeds per plant sown. The seeds were sown in plastic trays and transferred to the greenhouse where they were grown until young seedlings had emerged.

2.5 SNPs utilization for estimation of degree of cross-fertilization and paternal mating success

Leaf samples were taken from the young offspring plants. These leaf samples were then sent to the company, TraitGenetics in Gatersleben, Germany, to conduct SNP detection with the KASP assay of KBioscience (meanwhile called LGC Genetic).

Leaf sample harvesting procedure for SNP/KASP analysis is shown in figure 5. The detail procedure of leaf sample harvesting is followed. The plant sample kit which is used for KASP analysis includes 96 well plates. The youngest and fully opened leaf of each plant was taken as source of a leaf piece with approximate dimensions of 1 x 1 cm^2, this leaf pieces were inserted into one of the wells. After taking such samples from all sown seeds (young offspring) of one mother plant, the forceps were cleaned with ethanol to avoid any DNA contamination. Additionally, one well per plate was left without a sample to be used as control, with a different position on each plate. When all the wells were filled, the samples were kept in Styrofoam box with silica gel at the bottom of the box. After finishing taking all the samples, strip-caps (covers) were put onto the columns of the plate and the plates were then sent to company for SNPs analysis by KASP assay.
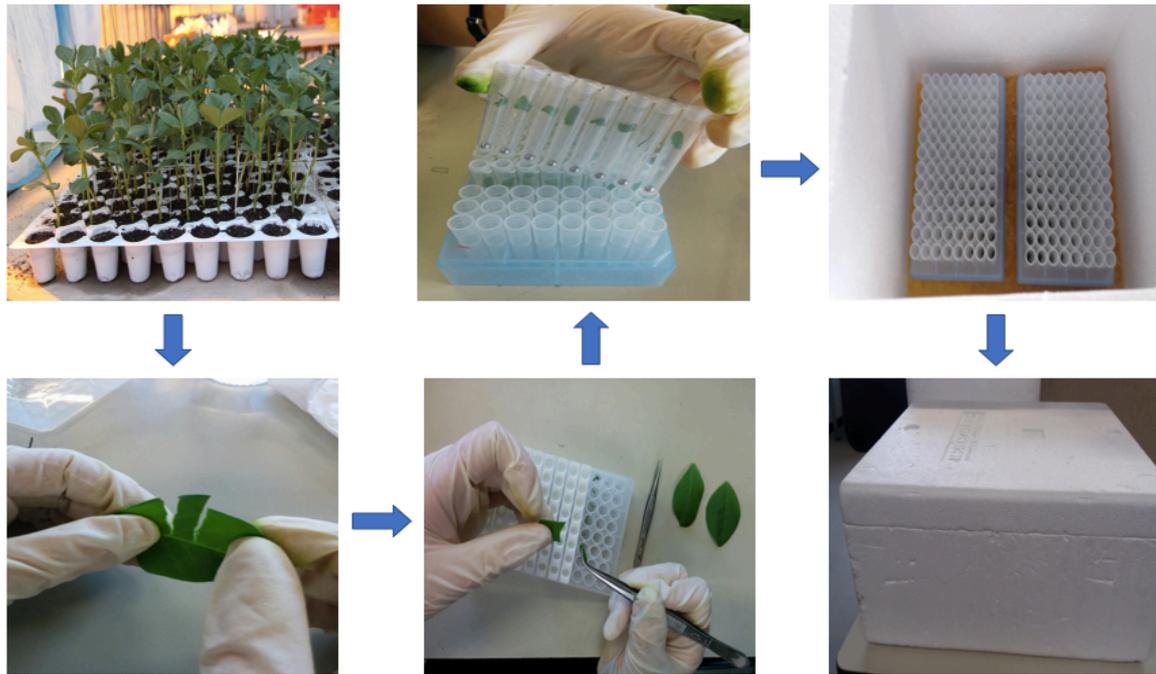
Figure 5. Leaf sample harvesting procedure for SNP genotyping. The leafs of seedlings which were grown in plastic trays are used for SNP analysis. The youngest opened leaf of each plant was sampled. After taking the leaf sample, put it into the respective well and strip-caps are used to closed that well. When plate was full, the microtiter plates were store without strip-caps on Silica gel in a Styrofoam box to prevent water condensation. At the end of a working day, the microtiter plates were capped again and sent to the company for SNPs analysis.

2.5.1 The Kompetitive allele-specific PCR (KASP)

There are three main components to achieve KASP; first, the DNA template (sample), second, KASP primer mixed and third, the KASP universal master mixed. The KASP primer mix contains two allele specific forward primers and one common reverse primer. Two allele specific primers are complementary with the targeted DNA sequences and each of them have a different tail sequences. The universal primer mix contains two oligo nucleotides, the KASP Taq polymerase and other components for the PCR. The first oligo nucleotide is called FAM, which is identical to the tail of the first forward primer and the second one called HEX, which is identical to the tail of the second forward primer. The PCR is conducted by the combination of these three components. In addition, the three primers which are included in KASP assay mixed are also called non-label oligo nucleotides and the two nucleotide which are included in KASP master mixed are called label oligo nucleotides and they are carrying fluorescent dyes. In the master mix, the fluorescence of the oligo nucleotides is suppressed by a quencher.

The first step of KASP assay is the first round of PCR, denaturing of targeted DNA sequence and a competing of the two allele specific primers to bind the target sequence that is complementary to the primers. When one such primers already annealed to the target DNA at one single strand, it will elongate. The common reverse primer anneals to the complementary DNA strand. Then, the tail of annealed primers would be assisted the amplification of the target sequence. As an example, when the targeted SNP state was either Adenine or Cytosine, the first primer would be bind with C and the second with C or A at another tail of sequence. The second round of the PCR starts again with DNA. The common reverse primer attaches to

the sequence amplified in the first PCR and elongates and copies the tail sequences of the forward primers. Thus, a complement of the tail sequences will be generated in this step. In the third round of the PCR, the labelled oligo nucleotides bind to the complementary sequences generated in round two. Fluorescence occurs during thermal cycling and no longer quenched. The homozygous or heterozygous stated of the SNP can be identified based on which of the two dyes is fluorescing. A summary of this process can also be seen in figure 6.
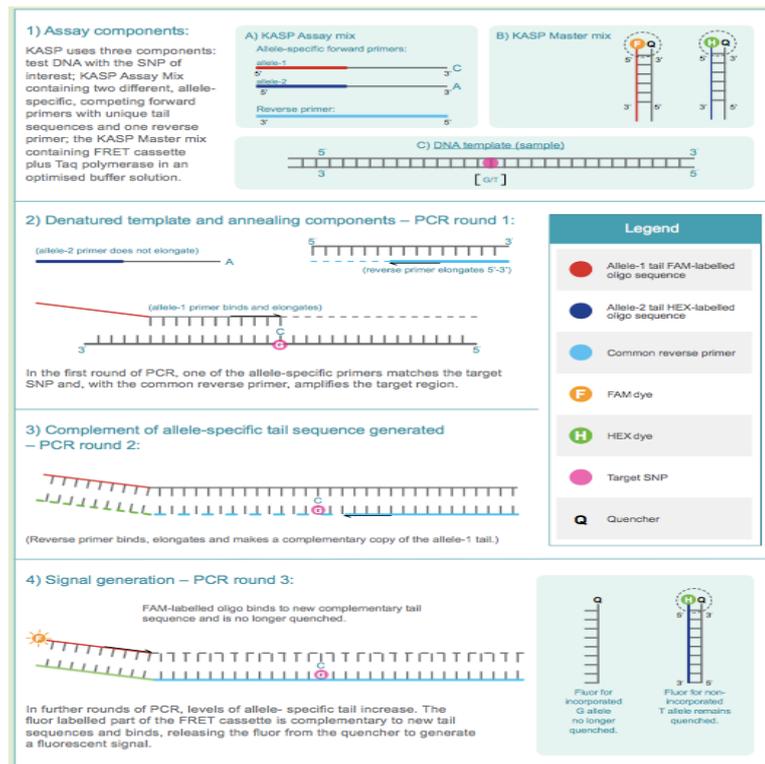
Figure 6. KASP reaction for two alleles (LGC, 2014). In this figure, a SNP with the two alleles G and T is analyzed. Two allele-specific forward primers and a common reverse primer are used to conduct an allele-specific PCR. The tails of allele-specific primers are identical to specific oligo-nucleotides (1A). These oligo-nucleotides are attached to fluorescent dyes so called FAM and HEX dye and they are attached to quenchers which quench the fluorescence when they are in the proximity of the dyes (1B). During the PCRs, the allele-specific forward primers anneal to the match SNPs and the target sequences and, reverse primer complete the tails that are generated in PCR round 1 and 2 (2 and 3). After the dyes are binding with complementary sequences, the fluorescence occurs and will no longer be quenched. PCR are continued until the SNP is showing the target sequence. Either this genotype is homozygous or heterozygous can be evaluated by the fluorescence generated by the dye.

In this work, ten selected SNP markers were used. The selected markers and detail information on their application are shown in Table 2.2. These SNPs were selected based on recommended 845 SNPs which individuals are validated for KASP assays according to Webb et al., (2016) by a consensus SNP-based genetic linkage map on six *Vicia faba* chromosomes. The selected SNPs were located on chromosome no. 1, 2, 3, 4 and 5 (Webb et al., 2016).

To distinguish between eight genotypes as possible pollen donors, only three SNP markers are necessary. However, we used eight SNP markers (1 to 8) to improve the validation. Markers 9 and 10 were used to check whether the plants of entries 4 and 5 were in fact F_1-plants derived from successful manual crossing or if the manual crossing failed and they

were in fact self-fertilized inbred lines. Those seeds of entries 4 and 5 that had been identified by markers 1 to 8 to be derived from self-fertilization were further investigated with markers 9 and 10. If the SNP result was homozygous then this seed had been growing on an inbred line and the manual crossing had been unsuccessful. Otherwise, if the SNP was heterozygous (TG or AG for entry 4 and GA or GT for entry 5), the manual crossing had been successful and that plant of entry 4 or 5 was in fact an F1 plant.

Table 2. Selected SNPs to identify the pollen donor of each analyzed offspring plant.

	SNPs	1	2	3	4	5	6	7	8	9	10
Entry	**Genotype**	Vf_Mt1g08998 0_001	Vf_Mt2g01097 0_001	Vf_Mt2g06488 0_001	Vf_Mt4g00703 0_001	Vf_Mt5g01359 0_001	Vf_Mt6g07121 0_002	Vf_Mt7g07880 0_001	Vf_Mt8g02080 0_001	Vf_Mt4g08589 0_001	Vf_Mt4g12510 0_001
1	**S_85**	C	T	G	A	C	G	G	G	A	T
2	**S_46**	T	C	A	A	T	A	A	A	A	T
3	**S_235**	T	T	A	A	T	G	A	G	A	T
4	**F$_1$(S_19xS_35)**	T	C	G	A	T	A	G	G	R	K
5	**F$_1$(S_25xS_217)**	T	T	G	C	T	G	A	G	R	K
6	**WAB_Fam157**	C	T	A	A	C	A	A	A	A	T
7	**S_145**	T	C	A	A	C	G	A	G	A	T
8	**S_199**	C	T	A	C	C	G	G	A	A	T

Source: Webb et al (2015) and Cottage et al., (2012). A for Adenine, T for Thymine, C for Cytosine and G for Guanine. These A, T, C and G show the specific SNP polymorphism of the corresponding genotypes. In this table C = CC, G= GG, T= TT, R= AG, K= GT based on ICUPA code system (ANNEX 2).

2.6 Calculating the degree of cross-fertilization and paternal mating success

All of the data used to conduct this research were kindly provided by Lisa Brünjes, Plant Breeding Institute, Georg-August-Universität, Göttingen. The identification of father genotype base on the result of SNPs was done with Python programming 2.7 (provided by Lisa Brünjes).

2.6.1 Calculating the degree of cross-fertilization (DOC)

Microsoft Excel 2010 program was used to calculate the degree of cross-fertilization. Having identified the father genotype for each seed sample, we were able to differentiate between self- and cross-fertilized seeds. Based on these values, the average degree of cross-

fertilization(DOC) values for each mother genotypes per block was calculated. The degree of cross-fertilization was calculated by using the following formula:

$$\text{Degree of cross-fertilization(DOC)}= \frac{\text{no. of cross-fertilized seeds per mothergenotype}}{\text{Total no. of analyzed seeds per mother genotype}}$$

2.6.2 Calculating the paternal mating success (PMS)

The paternal mating success (PMS) values for every single father genotype were calculated based on the same data set. The data were rearranged by block, father genotypes, total no. of seeds of mother genotypes which crossed with specific father. Subsequently, the absolute frequency of the different father genotypes per mother genotype per block was calculated. The mean value of paternal mating success per father per mother line and block was calculated by this equation:

$$\text{Paternal mating success(PMS)}= \frac{\text{No. of seeds from one specific mother crossed by one particular father}}{\text{Total no. of cross-fertilized seeds of one specific mother}}$$

2.6.3 Calculating the neighborhood proximity

Since the "complete neighbor balance" design was used, each mother genotype has the same frequency of being a neighbor to all different father genotypes across the polycross. However, the frequency of being neighbor of different father genotypes to be neighbor with specific mother genotypes was different for every single block and only balanced when looking at the entire polycross, across all eight blocks. Whether the proximity between one mother genotype and one father genotypes does influence the paternal mating success of those genotypes or not was a question in this work. Therefore, the horizontal, vertical and diagonal proximity of different father genotype individuals was counted separately and individually, based on adjacent neighbors. To know this, data were modelled based on the special pattern of the experimental design. The calculation was based on Pythagorean theorem. The horizontal and vertical proximity for single mother plant with a directly neighbor father plant was assigned as value "1" and any diagonal proximity was assigned as value 0.71. In the case of diagonal adjacent neighbor, the actual value by applying Pythagorean theorem was "$\sqrt{2}$". However, this value is a distance not proximity, and was not valid for the assumption that the higher proximity between two genotypes does higher chance of paternal mating success. Hence "$1/\sqrt{2} = 0.71$" was employed. This procedure

was applied for every single plant of the same genotype per block. All proximity values for one mother genotype and all fathers were summed up for single individual genotypes per block and for every genotype (Figure 7).
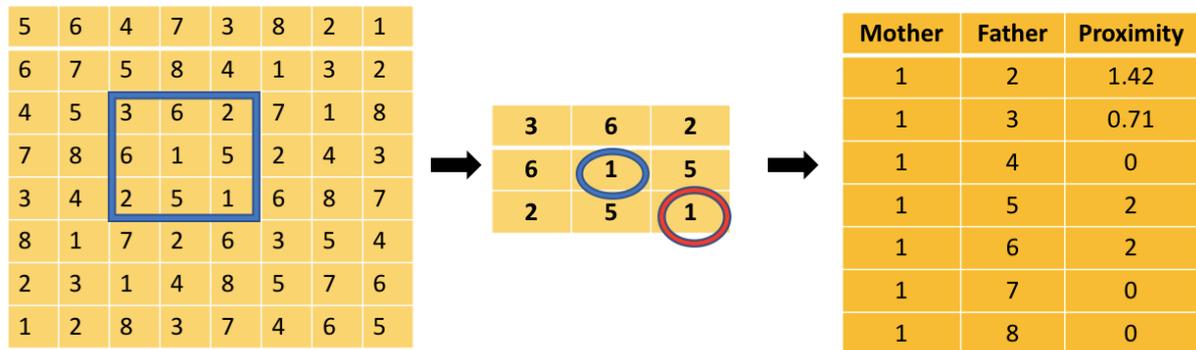
Figure 7. The calculating procedure for the proximity between one mother genotype and different potential father genotypes, which were being as adjacent neighbor. If the neighbor plant was located either horizontally or vertically adjacent, their proximity was assigned as 1. If the neighbor plant was located in diagonally adjacent, their proximity was assigned as 0.71. And multiply with the frequency of 1 and 0.71 respectively for the same genotypes per single plant and summed up the proximity of the same mother genotype with different father genotypes per block. In this figure, the single plant of entry 1 has 8 adjacent neighbor plants. Among them, one plant is the same entry and this plant is not considered as neighbor plant because it does not have a potential to cross-fertilize.

2.7 Data analysis

Statistical analysis and descriptive statistic were performed by using R version 3.1.1 (2014-07-10) and ggplot 2 package (Wickham, 2009).

2.7.1 ANOVA (Analysis of Variance)

Analysis of variances were carried out for the obtained data. The analysis was performed with the standard package of R. The pre-conditions to conduct a valid ANOVA include independent and normally distributed experimental error as well as homogeneity of the variances between the different populations of the residuals (Gomez and Gomez, 1984). The QQ (quantile-quantile) plots and Shapiro-Wilk test were additionally tested to derive the distribution assumption (Mardan, 2004; Shapiro and Wilk, 1965).

2.7.2 Multiple comparison among means

Multiple comparisons between mean were conducted after conducting ANOVA, if it showed a significance F-value. For that purpose, Tukey's test for the honest-significant distance (Tukey-HSD) as a post-hoc test for multiple comparisons of the means was used. This Tukey-HSD was designed for data analysis that have equal numbers of variables (Steven, 1999). Parts of the R script to carry out ANOVA and multiple mean comparison were written by Rebecca Tacke, Division of Plant Breeding, Department of Crop Sciences, Georg-August-Universität, Göttingen.

2.7.3 Correlation coefficient

Correlation coefficients between degree of cross fertilization and paternal mating success were calculated. Likewise, the correlation between proximity of mother and specific father genotypes and paternal mating success were calculated according to Pearson correlation method to address whether the neighborhood proximity between a mother and father plant may influence the paternal mating success per block.

3. Results

In this polycross sixty-four plants of one genotypes were planted in total. However, there were some environmental effects on the polycross and experimental error while the polycross was grown. During the flowering stage, 511 plants were standing. Hence, some of the genotypes does not have exactly sixty-four single plants during harvesting. We aimed to obtain 12 samples from each single plant for SNP analysis. Therefore, 12 to 14 samples per each single plant were taken and the mean sample is 96 per genotype per block. However, in 19 out of 512 plants, this was not possible. In 19 plants, there were between 228 and 266 samples because there were too few seeds or the seeds did not germinate. In 228 samples, the father genotype could not unambiguously be identified. As consequences, there were differences in identified data quantity for each genotype.

Analyzed data were shown in ANOVA by using linear regression model. For descriptive statistic, bar plots, box plots and scatter plots were used. Box plots were used to describe the data distribution at five distinct levels; lowest value, first quartile (25% of the data), medium of the data set, third quartile (75%of the data) and the highest value of the data set. If most of the data are belonging to the box, which means 25 to 75% of the data are closed to medium of data set, it can be concluded these data are normally distributed and are homogeneous.

Scatter plots also called x, y plots, were used to visualize any correlation between two variables in linear function. The correlation of two variables can be seen by the scatters distribution inside the scatter plot and their positive or negative correlation can be observed by correlation coefficient, r value.

3.1 Degree of cross-fertilization

The degree of cross-fertilization (DOC) was calculated as ratio of the number of crossed seeds to all seeds of each genotype per block (see section 2.7). As described above, there were differences in the data quantity that can identify the crossed father genotypes of individual mother genotype in this polycross. The identified data quantity of eight genotypes ordered by their respective degree of cross-fertilization is shown in figure **8**. The highest identified data quantities were found at entries 5 and 3 whereas entries 7 and entry 1 possessed the lowest identified data quantity. The results for degree of cross-

fertilization of these eight genotypes are shown in figure **9**. In that figure, box plots revealed the corresponding degree of cross-fertilization of each genotype.
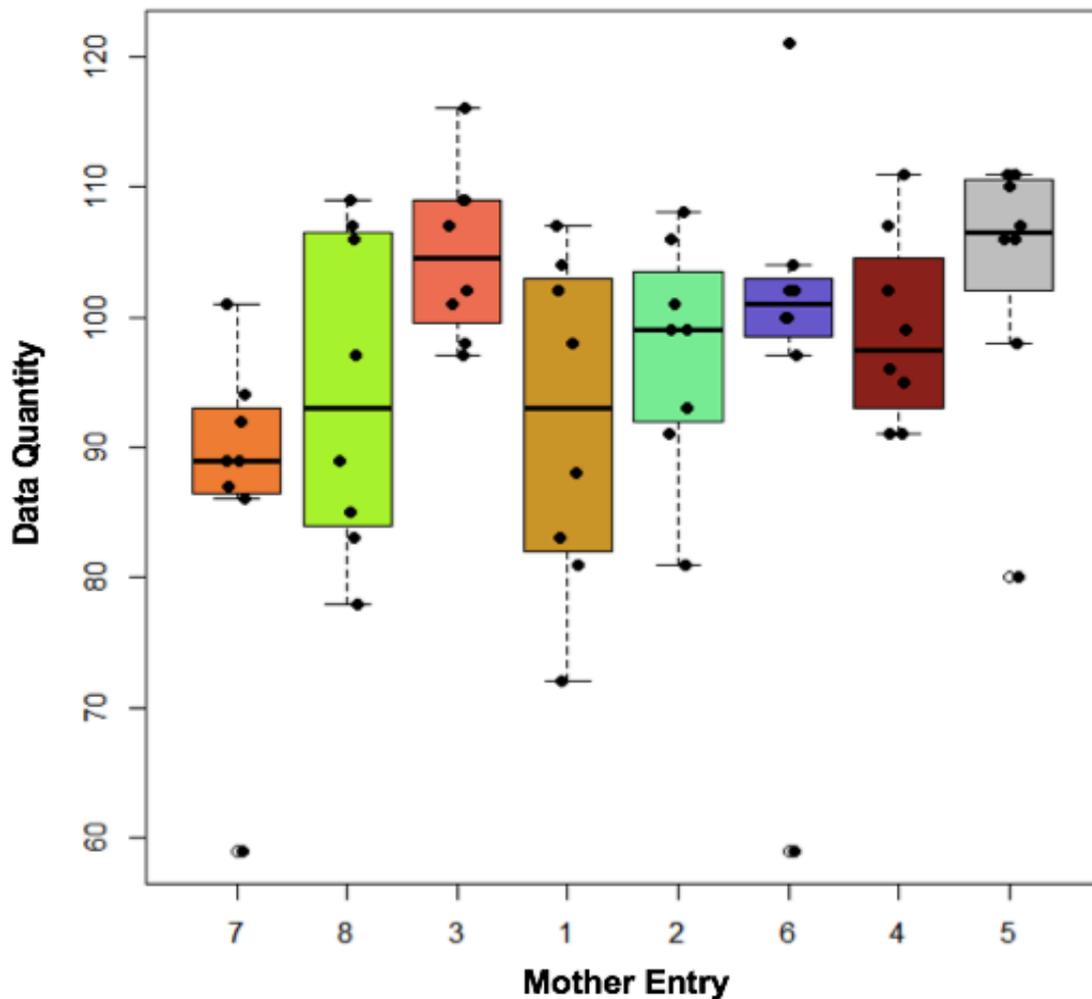
Figure 8. Analyzed sample data quantity to identify father per mother genotypes (entry 1 to 8) for polycross. The black dots on each box represent every single block. They are slightly scattered to avoid individual overlapping of points.

Entry 7 had the highest degree of cross-fertilization among these genotypes and the mean degree of cross-fertilization is 0.52(52%), followed by entry 3 with a mean value degree of cross-fertilization of 0.41(41%) the. The lowest means of degree of cross-fertilization were performed by the two F_1s, entries 4 and 5. Their mean degree of cross-fertilization was 0.25 (25%) and 0.13(13%) respectively.

Table 3. **Means of cross-fertilization degrees of the eight mother genotypes (entries).**

Entry	1	2	3	4	5	6	7	8	Overall mean
Mean	0.38	0.38	0.41	0.25	0.13	0.36	0.52	0.38	0.35

Population mean value of degree of cross-fertilization across eight genotypes was 0.35 (35%) (Table 3).
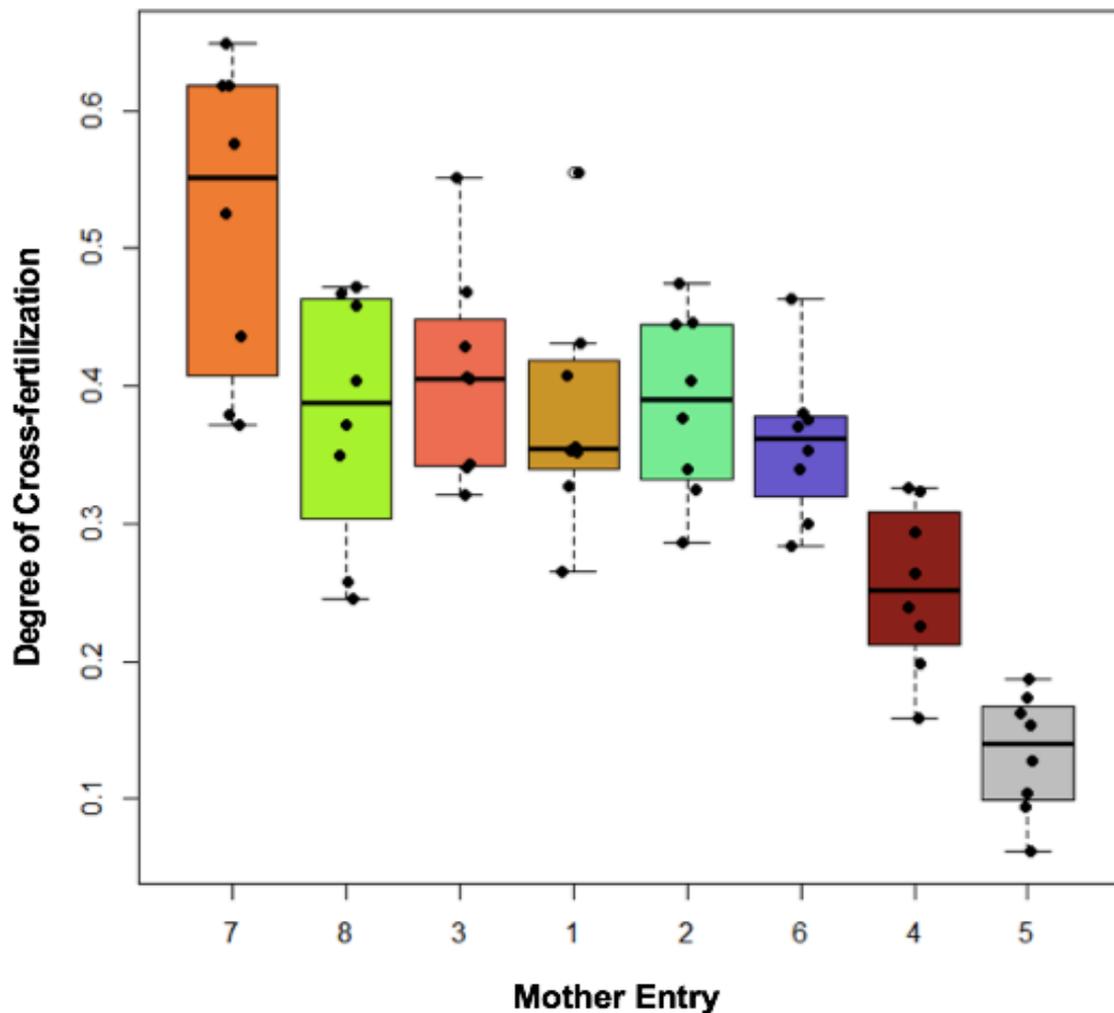
Figure 9. Degree of cross-fertilization across eight genotypes (entry 1 to 8) of polycross. Black spots on each box represent the variation of DOC across eight blocks. Mother entries (genotypes) are arranged according to descending order of degree of cross- fertilization.

An ANOVA was conducted to analyze whether any of the modelled source of variation (factors) showed any significant influence on the degree of cross fertilization. Mother genotypes and blocks were set as factors and their interactions as error term. For the 8

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different mother genotypes and the 8 different blocks of the polycross, the following linear model was constructed:

$$Xij = \mu + Mi + Bj$$

$Xij\ =\ observation\ of\ degree\ of\ cross-fertilization, i\ in\ mothers, j\ in\ blocks$

$\mu\ =\ general\ mean$

$M\ =\ effect\ of\ mother\ genotype\ i$

$B\ =\ effect\ of\ block\ j$

Hypothesis of Mother genotypes affected on degree of cross-fertilization were followed:

Null Hypothesis, H0.1: $\mu1=\mu2=\ldots\ldots=\mu8$

Alternative Hypothesis, H1.1: not all means are the same

Hypothesis of block effect on degree of cross-fertilization was followed:

Null Hypothesis, H0.2: $\mu1=\mu2=\ldots\ldots=\mu8$

Alternative Hypothesis, H1.2: not all means are the same

The outcome of the ANOVA is presented in Table 4.

Table 4. ANOVA for effect of mother genotype and block on degree of cross-fertilization.

Source of Variations	d.f	Sum of Square	Mean Square	F-value	p- value
Mother	7	0.74	0.11	18.15	1.203e-11 ***
Block	7	0.04	0.01	1.07	0.39
Error	49	0.28	0.01		

***, significant at 0.001 level.

The effect of mother genotypes on the degree of cross-fertilization was significant at a significant at 0.001 level. Hence the null hypothesis has to be rejected. It means at least one mother genotype was significantly different in degree of cross-fertilization from one other genotype. Block effect on degree of cross-fertilization was not significantly different from zero with a p-value of 0.3941. Therefore, the H0.2 (Blocks means are not different from each other) cannot be rejected.

Since the ANOVA is showing significant difference for at least one pair of mother genotypes, the Tukey test was conducted to check mutual significances among all pairs of

mother lines. A Tukey Test was done including p-value adjustment after Bonferroni to correct for multiple comparisons (Figure 10). Several genotypes were significantly different from each other in their degrees of cross-fertilization. These include mostly one of the genotypes 4 and 5.
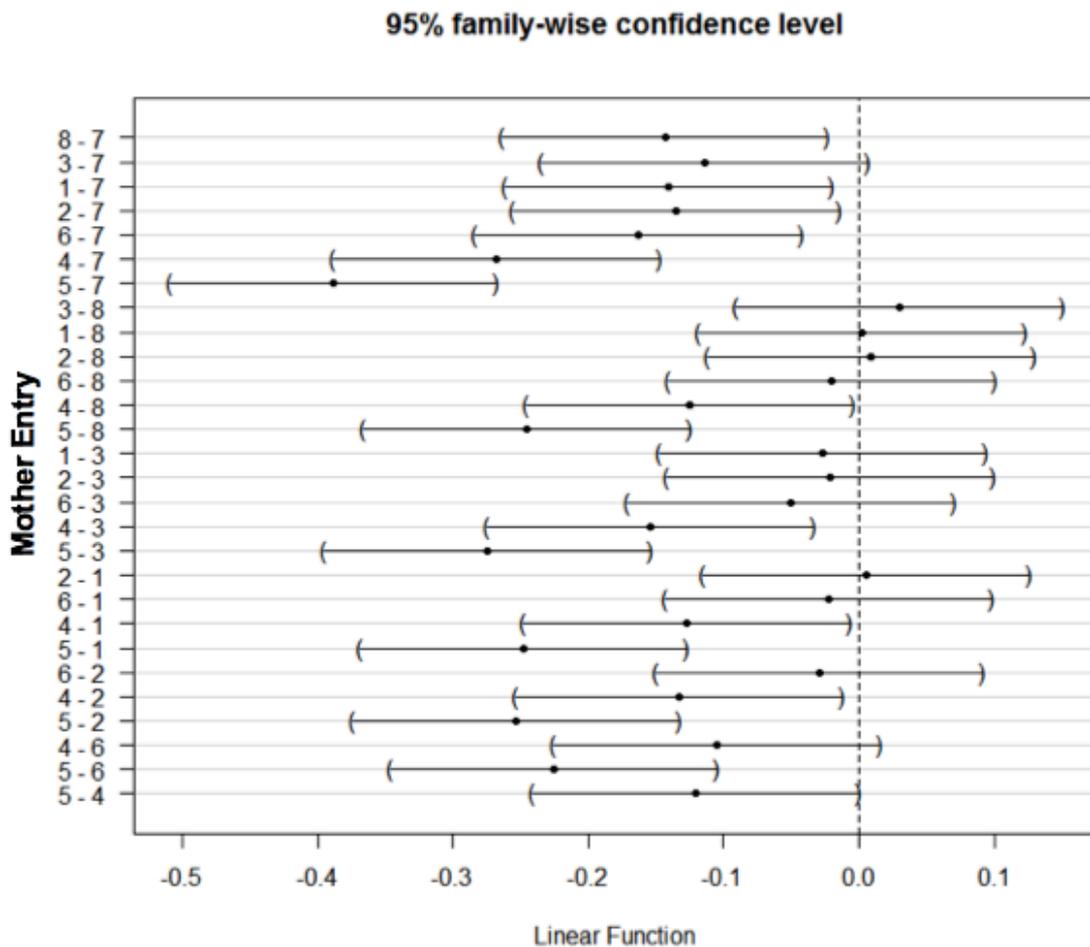
Figure 10. 95%-confidence intervals of results of Tukey Test of differences between means of cross-fertilization degrees of mother genotypes (entry 1 to 8). Each genotype was tested against each other genotype. The mother genotypes were tested against each other. The farther the value from the vertical significant line, the more significant is the difference between those genotypes.

3.2 Paternal mating success

Paternal mating success (PMS) values were calculated based on father genotypes per block (see section 2.7). The mean paternal mating success of eight genotypes and overall mean across these eight genotypes are shown in table 5.

Table 5. Means of paternal mating success of the eight father genotypes (entries).

Entry	1	2	3	4	5	6	7	8	Overall mean
Mean	0.13	0.17	0.08	0.27	0.26	0.09	0.07	0.06	0.14

Figure **11** shows the paternal mating success values of father genotypes in this polycross. The figure was arranged according to the mean values of paternal mating success, highest to lowest values in order. The highest amount of paternal mating success was occurred in entries 4 and 5 and followed by entry 2. Entry 7 and 8 had the lowest amount of paternal mating success values among these genotypes. The total paternal mating success of the eight genotypes is shown in figure 11.
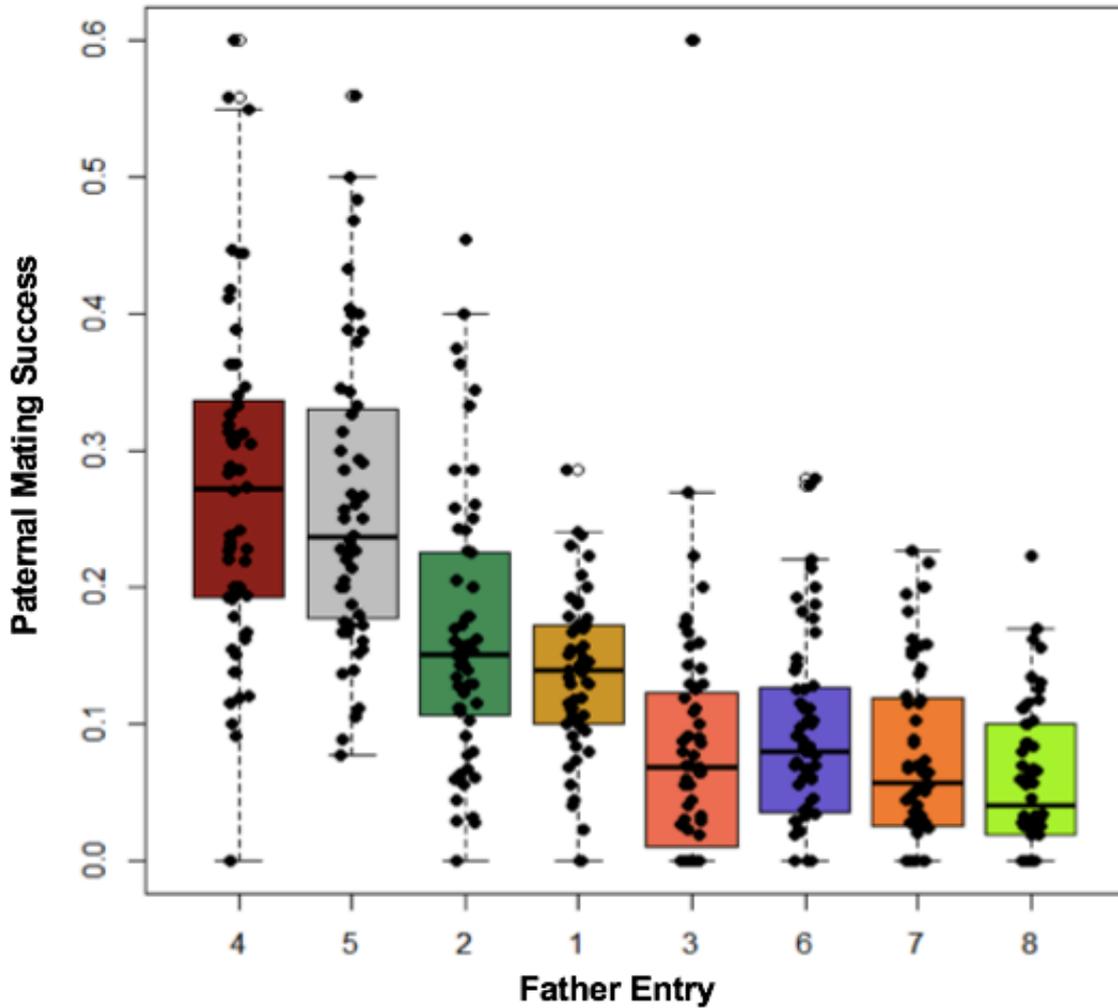
Figure 11. Paternal mating success across eight father genotypes (entry 1 to 8). Black spots on each box represent the variation of PMS of eight values for each of seven mother genotypes from eight blocks. Box plots are arranged according descending order of paternal mating success.

Figure **12** shows the total amount of paternal mating success for each father genotypes per mother genotypes. The contributions of entry 4 and 5 were higher than those of the other entries and lowest contribution in paternal mating success occurred in entries 7 and 8. Entries 4 was relatively successful in paternal mating success on entry 5, when compared to the other entries, and vice versa. It seems that mother and father interactions between these genotypes can influence the paternal mating success to some extent and this assumption was considered as a hypothesis in the linear model.
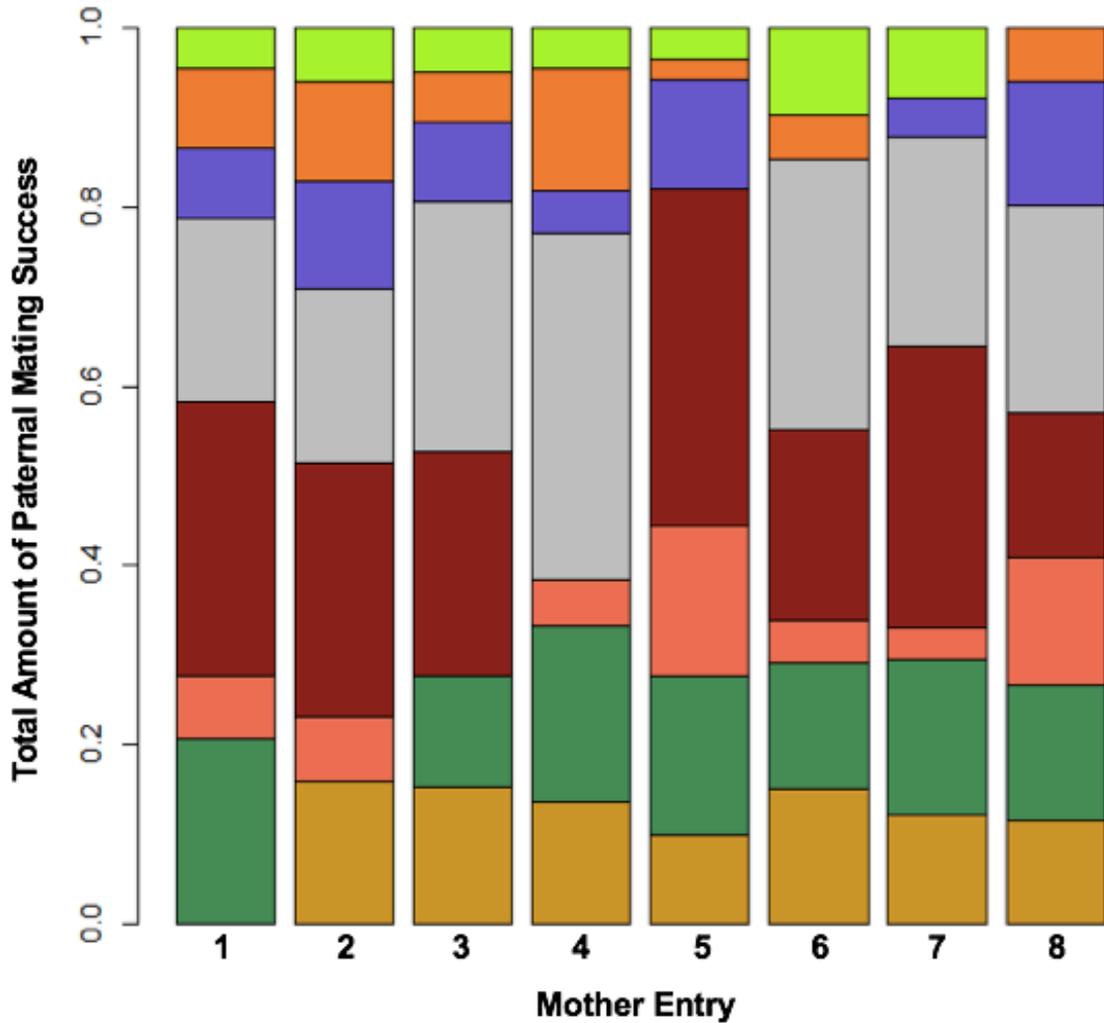
Figure 12. Amount of paternal mating success per mother genotypes based on crossed-fathers.
Golden, sea-green, coral, dark-red, grey, slateblue, chocolate and green colors represent the
respective amount of paternal mating success of crossed fathers (entry 1 to 8) upon each
mother genotype.

To investigate whether there are some factors that can influence the paternal mating success, ANOVA was conducted by using linear model. The different father genotypes, mother genotypes, father-mother interactions and blocks were considered as factors and the following linear model was constructed (with Mother x Father x Block triple interaction as error term):

$$Xijk = \mu + Mi + Fj + Bk + MFij$$

Xijk= observation of paternal mating success of mother genotype i, father genotype j in block k

$\mu = general\ mean$

$M\ =\ effect\ of\ mother\ genotype\ i$

$F\ =\ effect\ of\ father\ genotype\ j$

$B\ =\ effect\ of\ block\ k$

$MFij\ =\ mother\ i\ times\ father\ j\ interaction\ effect$

Hypothesis of Mother genotypes on paternal mating success was followed:

Null Hypothesis, H0.1: μ1=μ2=……=μ8

Alternative Hypothesis, H1.1: not all means are the same

Hypothesis of father genotypes on paternal mating success was followed:

Null Hypothesis, H0.2: μ1=μ2=……=μ8

Alternative Hypothesis, H1.2: not all means are the same

Hypothesis of block on paternal mating success was followed:

Null Hypothesis, H0.3: μ1=μ2=……=μ8

Alternative Hypothesis, H1.3: not all means are the same

The outcome of the ANOVA conducted with R can be seen in Table 5.

Table 6. ANOVA for the effect of father, mother, block and father: mother relations on paternal mating success.

Source of Variation	d.f	Sum of Squares	Mean Square	F-value	p-value
Father	7	2.8	0.4	58.4	< 2.2e-16 ***
Mother	7	0.1	0.0	1.2	0.3
Block	7	0.0	0.0	0.0	1.0
Father: Mother	41	0.8	0.0	2.7	3.564e-07 ***
Error	385	2.6	0.0		

***, significant at p- value 0.001 level.

The effects of father genotypes and of mother: father on paternal mating success were highly significant and the null hypothesis was rejected. It means that mean values of paternal mating success of at least one father genotype was significantly different from the mean value of at least one other father genotype. The effect of father: mother interactions on paternal mating success was also significant at 0.001 level. The alternative hypothesis, H1.2 and H1.3 were accepted. Block effects on paternal mating success were not different with a p-value of 1. Therefore, the eight blocks did not significantly influence the paternal

mating success in this polycross and therefore the null hypothesis H0.3 was accepted. The null hypothesis H0.1 for mother genotypes was also accepted because of there was no significant differences between mother genotypes.

Since the ANOVA had shown highly significant difference for at least one pair of father genotypes, Tukey test was conducted to analyze the different paternal mating success of every father lines against every other one. Tukey Test was done by p-value based on Bonferroni adjustment. Several genotypes were significantly different from each other in their degrees of cross-fertilization (Figure 13). These include mostly one of the genotypes 4 and 5.
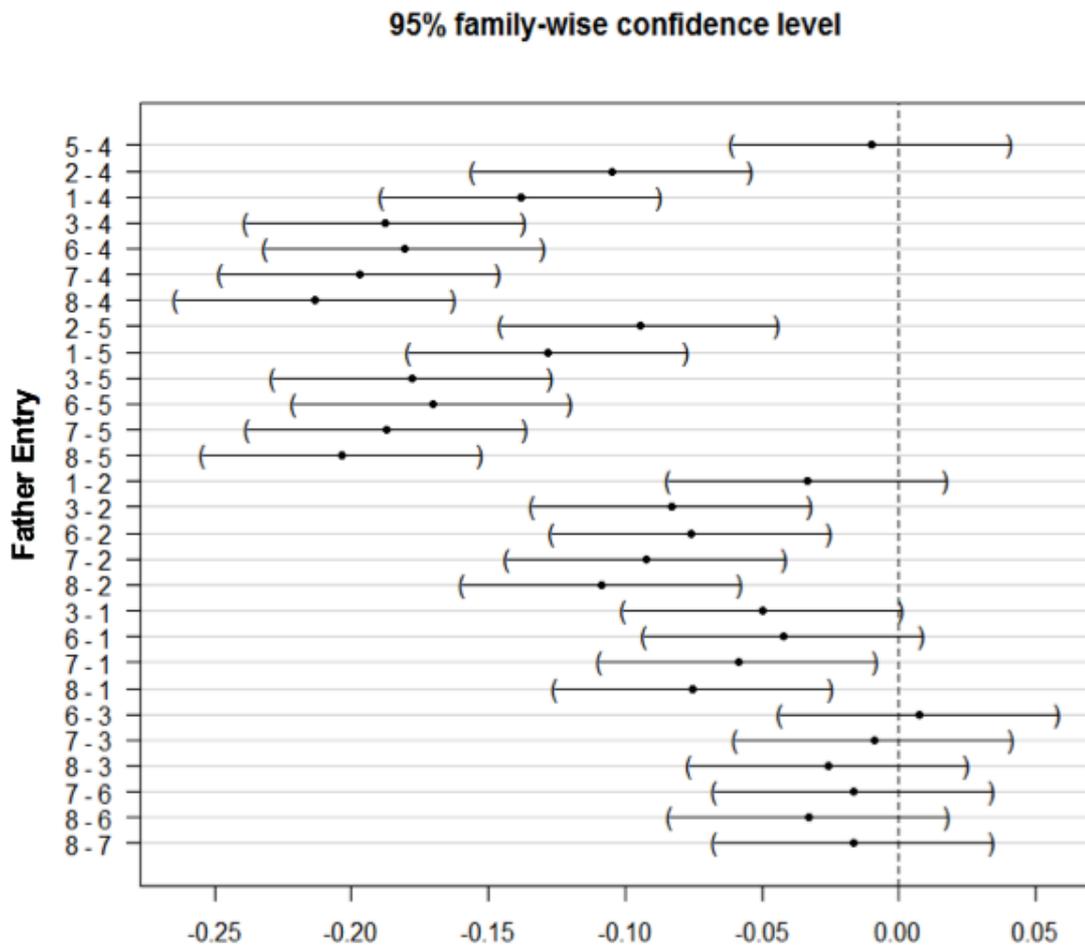
Figure 13. 95%-confidence intervals of results of Tukey Test of differences between means of paternal mating success of father genotypes. The father genotypes were tested against each other and the farther from significant lines mean the more significant different of those genotypes.

3.3 Correlation between degree of cross-fertilization and paternal mating success

The genotypes which had the highest degree of cross fertilization were shown the lowest paternal mating success values in figures **9 and 11.** Therefore, Pearson correlation was conducted to know whether these two variables have correlation. The following equation was constructed for these two variables.

$$r = \frac{\Sigma\,(xy)}{\sqrt{(\Sigma\,x^2)\,*\,(\Sigma\,y^2)}}$$

r = correlation coefficient

x= degree of cross-fertilization

y= paternal mating success across genotypes

$\sum$(xy)=covariance of x and y

Figure **3.7** shows the outcome of correlation coefficient value of -0.827 for the eight genotypes, significant at 0.05 level, which means that these two variables have strongly negatively correlated each other. However, if the F_1 genotypes are excluded, the correlation coefficient between these two variables for six inbred lines is only -0.335 and not significant.
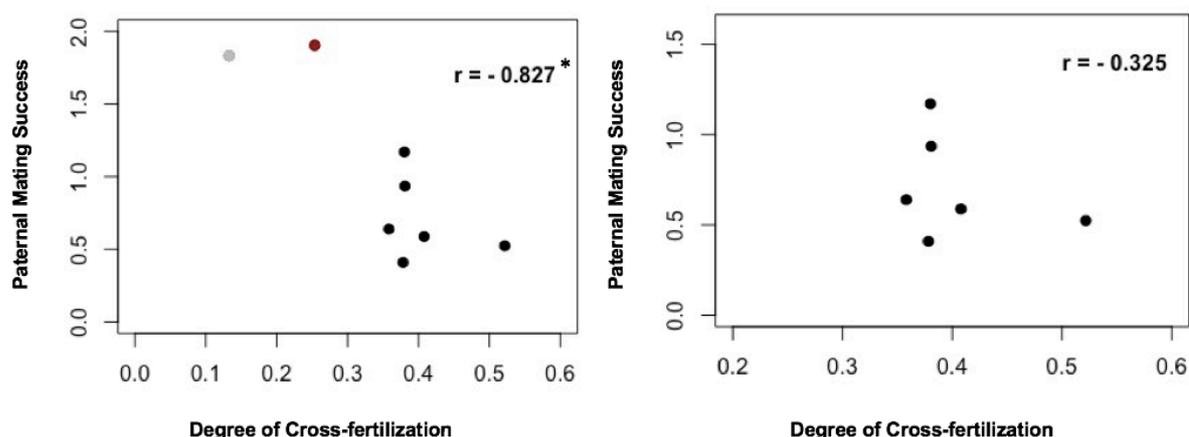
Figure 14. Correlation between degree of cross-fertilization and paternal mating success of eight genotypes: (a) Correlation for all eight genotypes and (b) Correlation for six lines, excluding F1 genotypes. Every single dot represents a different genotype.

3.4 Correlation between neighborhood proximity and paternal mating success

All proximity values between one specific mother genotype and all different fathers were summed up for single individual genotypes per block and for every genotype. Those neighborhood proximities between one genotype and different specific genotypes for each block were used for descriptive statistics to achieve the correlation coefficient between neighborhood proximity and paternal mating success.

The following equation was constructed for these two variables.

$$r = \frac{\Sigma\,(xy)}{\sqrt{(\Sigma\,x^2)\,*\,(\Sigma\,y^2)}}$$

r = correlation coefficient

x= neighborhood proximity

y= paternal mating success across genotypes

$\Sigma(xy)$=covariance of x and y

Although twelve to fourteen seeds per one single plant were regrown in order to obtain twelve successful result in the end, there were some plants samples where we were unable to distinguish which genotype was their father. Every genotype had the same chance of being neighbor of different genotypes across the polycross. Nevertheless, the frequency of being neighbor of one specific genotype with different genotypes was different for every single block. Therefore, the data quantities per block were different. Block 5, 6 and 4 had the three highest amount of data quantity (mean values not shown). The lowest amount of data quantities was shown in block 1, 7 and 3.

Figure **15** shows scatter plots of NP and PMS and the respective correlation coefficient values for each block separately. According to these outcomes, there was almost no correlation between these two variables in blocks 1, 2, 3, 4 and 7. Block 5 and 6 had slightly positive correlation and block 8 had slightly negative correlation.
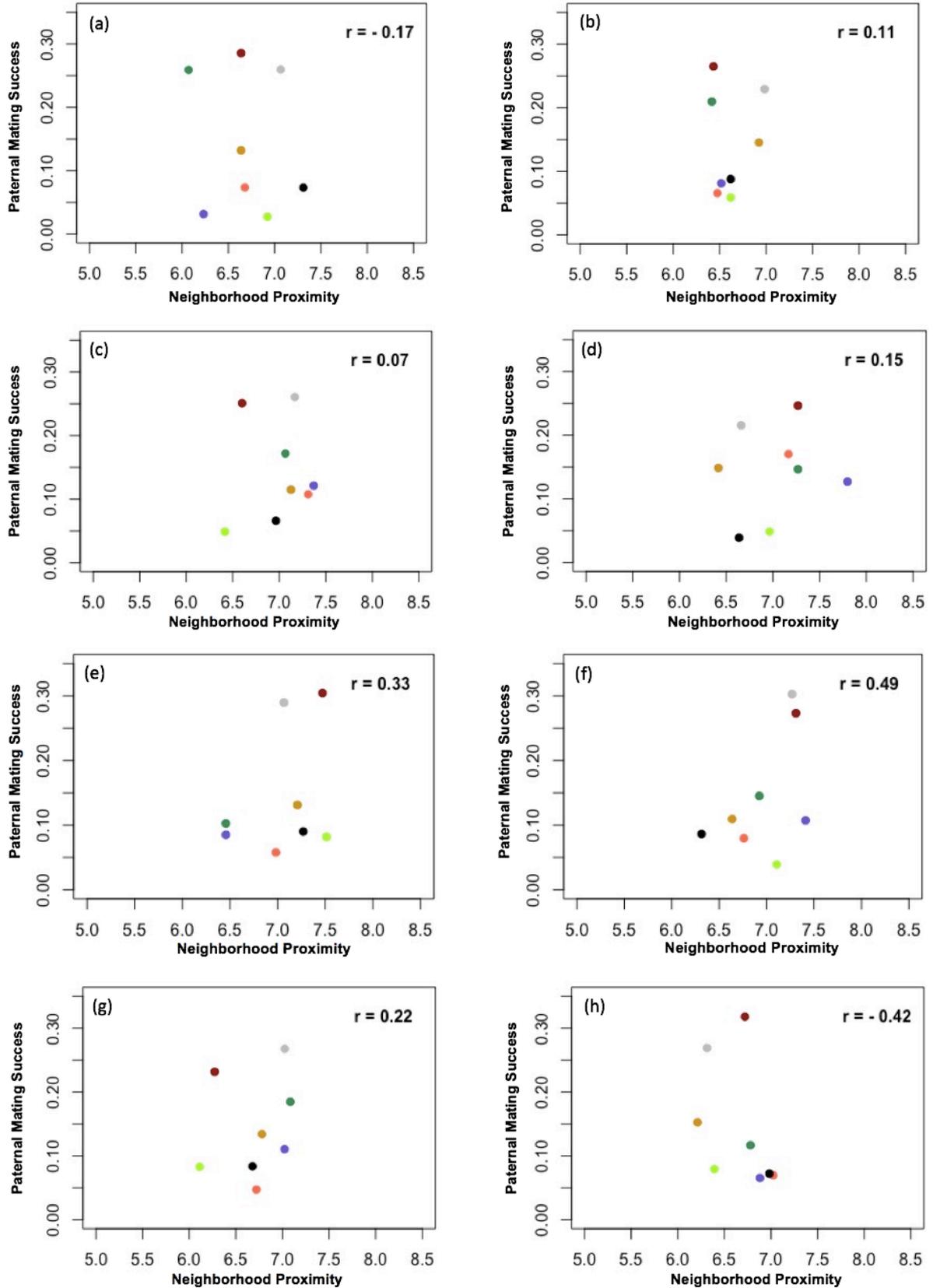
Figure 15. Correlation coefficient between paternal mating success and neighborhood proximity. Letters (a) to (h) represent blocks 1 to block 8. Every single point on each figure represent single genotype. Golden, sea-green, coral, dark-red, grey, slateblue, black and green points represent genotype 1 to 8, respectively.

Subsequently there was no correlation between NP and PMS when calculate caross all blocks, though **(Figure**. **16)**. Correlations between neighborhood proximity and paternal mating success were calculated (a) across all genotypes and (b) excluding F_1 genotypes. There was no correlation in any of the two situations.
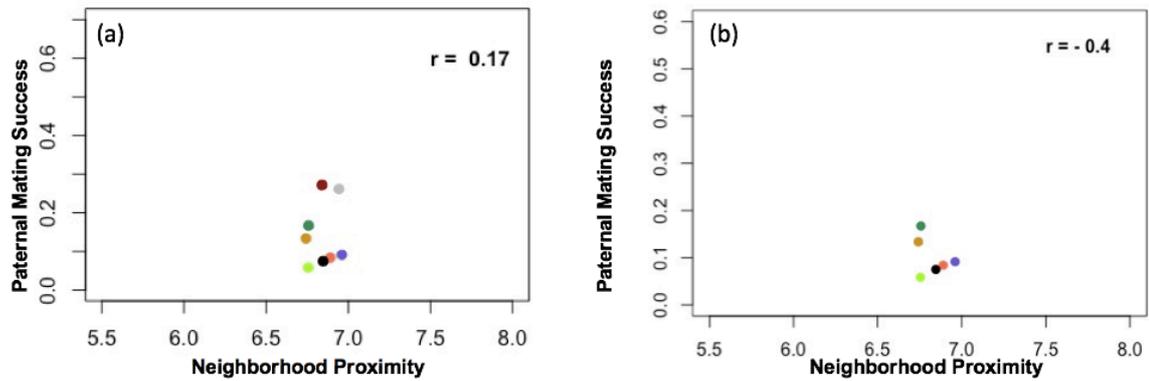
Figure 16. Correlation coefficient between NP and PMS. Figure a is the correlation coefficient including all genotypes of the polycross and figure b is excluding F_1s. Dark-goldenrod, sea-green, coral1, dark-red, grey, slateblue3, black and green-yellow points represent genotype 1 to 8 respectively.

4. Discussion

4.1 Degree of cross-fertilization

In this research, eight genotypes were grown in a polycross to study their degree of cross-fertilization. The average degree of cross-fertilization of all genotypes excluding F_1s was 40% and including F_1s was 35%. The mean degree of cross-fertilization of eight individual genotypes varied from 13% to 52%, population averages were not highly difference though (see table 2.1). The highest degree of cross-fertilization was achieved by entry 7(S_145) and the lowest by entry 5(F_1(S_25 x S_217)). The mean degree of cross-fertilization of entry 7 was 52% and it was highest degree of cross-fertilization among the mean value of all genotypes in this polycross. Link et al., (1994a) reported that an average degree of cross-fertilization of 53.5% for 36 faba bean lines evaluated in two years, which is larger than the highest value of any of the genotypes employed in the polycross. The degree of cross-fertilization of entry 7 was the highest among the eight genotypes, however, this value is not as high as the degree of cross-fertilization value of 70% founded by Bond (1987).

The degree of cross-fertilization for entry 8(S_199), 3(S_235), 2(S_46) and 1(S_85) were approximately the same mean (between 38 to 40%). Bond and Poulsen (1983) reported similar mean values of degree of cross-fertilization. The individual degree of cross-fertilization across eight blocks varied from 27% to 56%. This result is supported by the report of Suso et al., (1999) who stated that the degree of cross-fertilization can largely vary with genotypes. The entry 6(WAB_EP02_Fam157) performed the lowest mean degree of cross-fertilization among all lines. The average value of degree of cross-fertilization of six lines in this polycross varied between 36% and 56%. This finding is matching to the repeatedly reports for continental spring beans, where levels of 30%–60% have been reported (Metz et al., 1993; Link et al., 1994b).

The F_1s genotype 4(S_19 x S_35) and 5(S_25 x S_217) showed an average degree of cross-fertilization of 25% and 13% respectively. Additionally, entry 4 had nearly two times higher degree of cross-fertilization than genotype 5. A higher mean degree of cross-fertilization of F_1 genotypes than these two F_1 was reported by Gasim et al. (2004). The degree of cross-fertilization for entry 4 and 5 had ranged from 6% to 33% in the different blocks, nevertheless most of them were less than 20%. The mean degree of cross-fertilization varied from 5% to 25% was reported on four faba bean cultivars under three

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different locations (Gasim et al., 2011).

According to those outcomes, the inbred lines had higher degree of cross-fertilization than the F_1s in this polycross. There is a difference in inbreeding level between inbred lines and F_1 genotypes. Draynar (1959) stated that the higher degrees of cross-fertilization occur in inbred than in cross-bred. Moreover, this is in good agreement with a report by Link (1990), who states the more heterozygous a genotype is, the more will the cross-fertilization be reduced. Therefore, the results presented in this thesis are aligned with these two-scientific publications.

Link (1990) reported that autofertility is negatively correlated with degree of cross-fertilization. Although autofertility rates of these eight genotypes under this research was not analyzed, it can generally be assumed that they have different autofertility rates based on their degree of cross-fertilization while they were standing in polycross. The large variability for autofertility across these eight genotypes, especially for F_1 genotypes are promising for genetic improvement to develop cultivars that are less dependent on pollinators to realize high yielding (Robertson and El-Sherbeeny 1995).

Many authors reported repeatedly that the extend of cross-fertilization in faba bean can be used for heterosis exploitation in synthetic varieties and hybrids (Link, 1990; Stelling et al., 1994; Link et al., 1994; Embyer, 1998 and Zeid et al., 2004). To exploit heterosis in synthetic varieties, it should be done by selecting those patent genotypes which have higher degree of cross-fertilization. Therefore, entry 7 should be used as a parental line because it has the highest degree of cross-fertilization among these eight genotypes. On the other hand, extreme degree of cross-fertilization of *Vicia faba* L. genotypes all over the world was reported between 10% to 70% (Bond, 1987). Based on this report, the degree of cross-fertilization of another inbred line of this polycross were not very low. Those lines probably result in high degrees of cross-fertilization when they get the chance to cross with father genotypes which have higher pollen distribution rates. This issue will be discussed in section 4.2.

As mentioned in chapter 1, only hybrid breeding has the chance to fully exploit heterosis. In faba bean, heterosis for grain yield where F_1 hybrids more than both better parent and mid-parent means has been reported (Zeid et al., 2004). F_1 hybrids of this polycross had lower degree of cross-fertilization, though, they are heterozygous and might have higher heterosis for yield than their parental means and the means of inbred lines those were used

in this experiment.

The level of heterosis for yield and the degree of cross-fertilization are important for breeding of populations such as synthetic populations and hybrid breeding (Link et al., 1994). Therefore, it can get higher heterosis and higher degree of cross-fertilization in these F_1 genotypes if their parents have higher degree of cross-fertilization. However, heterosis for yield is not significant in F_2 and F_3 generation (Metz et al., 1993) even though their cross-fertilization rates increased by increasing homozygosity (Link, 1990). The CMS systems which would be a prerequisite for heterosis exploitation have the constraint that the pollen sterility in male sterile lines is not enough (Link et al., 1997). Without a stable CMS system, hybrid breeding in faba bean is not feasible due to high time consuming and high cost in production of manually crossed hybrid seeds.

The inbred lines used for this research might have an adequate autofertility rate with proper cross-fertilization to fulfill the breeding objective of synthetic varieties. Normally, synthetics faba beans are partially homozygous and heterogeneous and can perform with a markedly higher yield stability (Stelling et al., 1994). As parental line, an inbred line of faba bean with its degree of cross-fertilization has an influence on heterosis and inbreeding level in a synthetic, it properties can thus be heritable to the next generations (Link, 1990; Link et al., 1994a). Therefore, the selection of individual parent lines is important to improve the yield stability not only for synthetic varieties but also for F_1 genotypes.

The influences of agronomic characters should also be considered for degree of cross-fertilization apart from the genetic aspect. Regarding influences of biotic and abiotic factors, degree of cross-fertilization varied depending on the genotypes, environmental factors, row spacing and pollinator activities (Gnanasambandam et al., 2012). Since, faba bean is a partially allogamous crop, not only for cross-fertilization but also self-fertilization is mainly dependent on pollinators under natural pollination, i.e. without tripping for self-fertilization. Pollinator activities during flowering and the temperature during the flowering period are also important for fertilization. The identified data quantity of entry 7 was the lowest out of eight genotypes and the highest amount occurred in entry 5. However, this is coincident with the degree of cross-fertilization of these two genotypes and it cannot be influence the degree of cross-fertilization.

The paternal mating success will be discussed in next section, because is also important and can have influences on the genetic make-up of the synthetic (Tacke, 2017). Afterward,

the neighborhood proximity between mother and father genotypes can influence paternal mating success or not will be discussed in section 4.3.

4.2 Paternal mating success

Although many authors reported that the degree of cross-fertilization in faba bean is important for yield stability (see section 4.1), they were just considered from the side of mother genotypes. Concerning about the contribution of father genotypes on successful cross-fertilization is a new aspect which had so far only been discussed by Brünjes (2014) and Tacke (2017).

In this polycross, the paternal mating success of father entries 4 (F_1(S_19 x S_35)) and 5(F_1(S_25 x S_217)) were significantly higher than that of six inbred lines. The lowest paternal mating success values were performed by entry 7 and 8 which achieved the highest degree of cross-fertilization among eight genotypes (see figure 9 and 12). The paternal mating success was highest in the F_1 genotypes, followed by entry 2. The entries 1, 6, 3, 7 and 8 are all inbred lines and resulted in similar paternal mating success values (see table 5). Thus, entry 2 should be considered not only for mother genotype but also as a highly potent father for cross- fertilization by the higher paternal mating success among lines. According to those outcomes, the inbred lines had lower paternal mating success than the F_1s in this polycross.

Since there was a significant effect of some father and mother interactions on paternal mating success, the contribution of father entries 2, 4 and 5 might be part of this influence with some extend. The interactions between F_1 fathers and inbred mothers show higher paternal mating success than the interactions between inbred father and mother. The interactions between father 7 and mother 4 was the highest paternal mating success of entry 7 and the highest paternal mating success of entry 6 was occurred in the combination with mother 6. Entry 2 shows probably the same paternal mating success on F_1 mothers. Therefore, it can be concluded that, the interaction between heterozygous father and homozygous mothers can achieved higher paternal mating success. However, the statistical mean comparison for those interactions was not conducted due to time limitation and can only be seen in a bar plot (Figure 12). On the other hand, Metz et al., (1994) reported that the genotype of pollen donor cannot influence the cross-fertilization if there is flower synchronization in spring faba bean.

Pollen distribution and pollen acceptance of genotypes depends on the inbreeding level in this experiment. Figure 12 shows all of the six inbred lines accepted nearly the same amount of pollen from any different genotype. Among them, they accepted F_1s pollens rather than lines, which has the heterozygous condition. It was probably that F_1 genotypes had higher pollen distribution rates meanwhile the foreign pollen acceptance rates of six inbred lines were high rather than pollen distribution. Gasim and Link (2009) reported that pollen distribution rates and foreign pollen acceptation rates were different between four genotypes by using RFLP markers.

Since faba bean is an entomophilous species, pollinator activity is important for fertilization especially for the genotypes which have low autofertility rates. The preference of pollinator on different genotypes would not be the same, eg. due to differences in color or amount of nectar in the flowers. This could be another reason for F_1 fathers who had higher pollen distribution rates. However, whether F_1s pollens have either higher pollen fertility rates or pollen tube germination rates than pollen of inbred lines is not clear.

On the other hand, pollen quality limitation should be included for paternal mating success, i.e., the failure of foreign pollens to combine with the stigma of the flower of the same or different genotypes for successful cross-fertilization (Aizen and Harder, 2007) probably happened in these genotypes under open pollination.

Additionally, the genotypes which have a lower paternal mating success are suitable for heterosis exploitation of faba bean by contributing a particular amount of pollens for successful cross-fertilization. Therefore, the lines which had higher paternal mating success among six inbred lines could carry the further potential for genetic transfer with their high degree of cross-fertilization. F_1 genotypes should be considered as promising father genotypes as they can contribute to the successful cross-fertilization in faba bean.

The correlation between paternal mating success and neighborhood proximity of these genotypes will be discussed in next section.

4.3 Correlation between paternal mating success and degree of cross-fertilization

As described in section 4.1 and 4.2, higher degree of cross-fertilization values was found in inbred lines, whereas higher paternal mating success was found in F_1 genotypes. Therefore, Pearson correlation coefficient was conducted by using these two variables for

eight genotypes (Figure 14 a). The hypothesis was there will be some negative correlation between degree of cross-fertilization and paternal mating success of these eight genotypes. According to descriptive statistic, paternal mating success and degree of cross-fertilization of these eight genotypes shown a highly negative correlation across eight genotypes. However, there was another hypothesis, correlation between these two variables of six lines could probably show a different value, since inbred lines were shown to have the opposite trend regarding these variables when compared to the F_1 genotypes. Thus, correlation for these two variables across six inbred lines was conducted by Pearson correlation coefficient and the result shows an only slightly negative correlation. The six inbred lines had performed a higher degree of cross-fertilization and lower paternal mating success than F_1 genotypes and such there was a slightly negative correlation between these two variables. According to these outcomes, the more heterozygous the genotypes, the higher the negative correlation between degree of cross-fertilization and paternal mating success. This is an information for faba bean breeding programs to consider heterosis exploitation of faba bean by the contribution of father genotype.

4.3.1 Correlation between neighborhood proximity and paternal mating success

Correlation between neighborhood proximity and paternal mating success of these eight genotypes was described in figures 15 and 16. According to those results, there were slightly positive and negative correlations observed in some blocks. However, those correlations values were low and some blocks have shown no correlation. Therefore, correlations of mean values of these two variables across eight genotypes and excluding F_1 genotypes of this polycross were conducted and no correlations were achieved. It can be assumed that there is no correlation between neighborhood proximity and paternal mating success for this polycross. Therefore, the paternal mating success of the father genotypes is depended on the genotypes and it cannot be influence by the neighborhood proximity.

The neighborhood proximity data obtained from this model included the proximity of adjacent neighbors and a further model should also consider indirect neighborhood effects to verify the result of this study. Additionally, it should be conducted the correlation coefficient between paternal mating success on father and mother interaction and neighborhood proximity of those father and mother genotypes for the further study.

5. Conclusions and outlook

The six inbred lines had achieved the higher degree of cross-fertilization and the lower paternal mating success. The higher paternal mating success with lower degree of cross fertilization was observed in F_1 genotypes. A negative correlation of these two variables and no correlation between paternal mating success and neighborhood proximity of father genotypes were observed in this polycross. A higher negative correlation between degree of cross-fertilization and paternal mating success was occurred at the more heterozygous genotypes. The higher paternal mating success was performed by the combination of heterozygous father and homozygous mother. It can be concluded that the degree of cross-fertilization and paternal mating success are changing depending on the level of inbreeding and on differences of genotypes.

These outcomes make a suggestion to apply breeding of synthetic varieties by selecting few parents and that can exploit the heterotic effects with an enhanced outcrossing rate. The relevance of F_1 genotypes to exploit the heterotic effect in synthetic varieties is needed to be concerned. The results presented in this work open new perspectives for future faba bean breeding. However, the further studies are needed to validate this outcome for future faba bean breeding.

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Annexes

Annex 1. BBCH- identification keys

Code	Description
Principal growth stage 5: Inflorescence emergence	
50	Flower buds present, still enclosed by leaves
51	First flower buds visible outside leaves
55	First individual flower buds visible outside leaves but still closed
59	First petals visible, many individual flower buds, still closed
Principal growth stage 6: Flowering	
60	First flowers open
61	Flowers open on first raceme
63	Flowers open 3 racemes per plant
65	Full flowering: flowers open on 5 racemes per plant
67	Flowering declining
69	End of flowering
Principal growth stage 7: Development of fruit	
70	First pods have reached final length ("flat pod")
71	10% of pods have reached final length
72	20% of pods have reached final length
73	30% of pods have reached final length
74	40% of pods have reached final length
75	50% of pods have reached final length
76	60% of pods have reached final length
77	70% of pods have reached final length
78	80% of pods have reached final length
79	Nearly all pods have reached final length
Principal growth stage 8: Ripening	
80	Beginning of ripening: seed green, filling pod cavity
81	10% of pods ripe, seeds dry and hard
82	20% of pods ripe, seeds dry and hard
83	30% of pods ripe and dark, seeds dry and hard
84	40% of pods ripe and dark, seeds dry and hard
85	50% of pods ripe and dark, seeds dry and hard
86	60% of pods ripe and dark, seeds dry and hard
87	70% of pods ripe and dark, seeds dry and hard
88	80% of pods ripe and dark, seeds dry and hard
89	Fully ripe: nearly all pods dark, seeds dry and hard

Source: Weber and Bleiholder, 1990

Annex 2. IUPAC code

IUPAC Code	Meaning
A	A
C	C
G	G
T/U	T
M	A/C
R	A/G
W	A/T
S	C/G
Y	C/T
K	G/T

Declaration

Here with I assure that I have composed by myself the present paper, without any help from any other person and only with sources and auxiliary means explicitly indicated in the papers.

Also, parts verbally and analogously adopted from other papers are indicated.

I have taken due account of "Guideline of Good Scientific Practice" released by University of Göttingen.

Date: Signature: