

Genetic inheritance and identification of molecular markers linked to male sterility in African marigold (*Tagetes erecta* L.)

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Research Article

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Abstract

The demand for hybrid varieties of African marigold (*Tagetes erecta* L.) is on the rise due to the increased productivity and uniformity exhibited by F₁ hybrids. To simplify the hybridization process, the use of a male sterile line is essential, as emasculation can be complicated due to the unique flower structure of marigold. In this study, seed-propagated petaloid male sterile lines with a genetic inheritance were employed. The inheritance of sterility was investigated across six generations (P₁, P₂, F₁, F₂, BC₁ and BC₂) and confirmed a single dominant gene governing petaloid male sterility over multiple seasons. The identification of molecular markers closely linked to the male sterility locus is crucial for accurately identifying genotypes with the desired traits within breeding populations. The marker CPSSR-39 exhibited clear and consistent segregation, following a Mendelian 1:1 ratio in accordance with the genes governing petaloid male sterility. This study represents the first report of genic petaloid male sterility and demonstrates the validation of linked markers, highlighting the potential use of CPSSR-39 in marker-assisted selection in marigold. This breakthrough opens up numerous opportunities for the commercial hybrid seed production of marigold, marking a significant advancement in marigold breeding.

INTRODUCTION

Among flower crops, marigold (*Tagetes erecta* L.), a member of the Asteraceae family, is gaining popularity due to the rising demand for loose flowers in the market as well as an industrial input for the extraction of carotenoids. There is a global market for carotenoids as food colourants, animal feed, and nutraceuticals (Berman et al., 2015). Carotenoids are natural pigments with antioxidative properties that guard against oxidative stress (Sandmann, 2014). Some carotenoids are chemically synthesized, while marigold is the major natural source of carotene (Rodrigues et al., 2019) and hence area under cultivation of marigold has been increasing over the years, and so is the rise in demand for hybrid seeds of marigold.

Marigold flower has a capitulum inflorescence consisting of ray and disc florets. Functional anthers in marigold are hidden within disc florets in the centre of the flower, making emasculation a difficult process. Male sterility is the major approach to avoid emasculation to cut down the labour and cost of hybrid seed production and ensures varietal purity (Chen and Liu, 2014; Kim and Zhang, 2018, Du et al., 2020). In marigold, apetaloid and petaloid are the two types of male sterility reported. The absence of ray florets is associated with apetaloid male sterility (Gupta et al., 1999, He et al., 2009; Tejaswini et al., 2016a), while flowers with only ray florets are associated with petaloid male sterility (Tejaswini et al., 2016a).

In marigold, apetaloid male sterility is reported to be controlled by a single recessive gene (Gupta et al., 1999, He et al., 2009; Tejaswini et al., 2016a) while petaloids are reported to be cytoplasmic inheritance and maintained by vegetative propagation (Kumar et al., 2017, Tejaswini et al., 2016b). In the GMS system of apetaloid male sterility, the segregation occurs at a 1:1 ratio necessitating the removal of 50% fertile plants, which is time and labour-consuming and also difficult to identify prior to flowering. Identifying molecular markers tightly linked to the male sterility locus helps in the early and accurate identification of male sterile genotypes at the seedling stage itself (Hayashi et al., 2011, Naresh et al., 2018). The use of molecular markers linked to male sterility has been reported in crops such as rice (Wang et al., 2004), rapeseed (Hong et al., 2006) and lettuce Hayashi et al., 2011).

Marker systems such as SCAR (He et al., 2009) AFLP ((He et al., 2010) and SSR (Asha et al., 2019) linked to apetaloid sterility have been reported. Though sufficient work has been reported with apetaloid sterility in marigold, there is not much information available on seed-propagated petaloid male sterility either in terms of gene action or in terms of a molecular marker associated with that. Apetaloid flowers are not marketable as the petals are absent. Hybrids produced from petaloid male sterile lines exhibit good combining ability for increased flower weight and biochemical components compared to apetaloid sterile lines (Santosh et al., 2018). In this study, we tried to unravel the gene action associated with seed-propagated petaloid male sterility and the marker associated with that, in an attempt to widen the genetic base of male sterility in marigold.

MATERIALS AND METHODS

Plant materials for the present study were selected from the progeny population of an ongoing marigold breeding program at ICAR-Indian Institute of Horticultural Research, Bengaluru, India. Thousands of progeny plants resulting from distant hybridization and selected inter-breeding during 2010-2018 were screened. The petaloid male sterility governed by nuclear genes was fixed by intercrossing within male sterile lines and stabilized over generations during marigold breeding programme.

Plant material

Three male sterile lines viz., IIHRMOP 1111, IIHRMOP 22 and IIHRMOP 228, were used for the present experiment. Each one of these male sterile lines consisted of both fertile and male sterile plants. Intercrossing between sterile and fertile plants within the individual line was attempted along with the selfing of fertile plants of the same line. Observations on the number of fertile and sterile plants resulting from intercrossing as well as selfing were recorded in the F_1 generation, and a segregating ratio was worked out for each one of the three male sterile lines under study (Table 1). Line IIHRMOP 1111 is selected based on the stability in segregation. For confirmation of genes governing the sterility and to ensure the stability of the segregation, repeated intercrossing between sterile and fertile plants of the selected line IIHRMOP 1111, along with the selfing of fertile plants for three consecutive seasons, was performed. Observations on the segregation of fertile and sterile plants were recorded in progenies from intercrossing as well as selfing..

Development of F_1 , F_2 , BC_1 and BC_2 (Six generation mean analysis)

The petaloid male sterile line (IIHRMOP 1111) crossed with a fertile variety Pusa Narangi Gaiinda to derive F_1 , and F_1 s were further selfed to obtain F_2 s. F_1 s were backcrossed to obtain BC_1 (IIHRMOP 1111 $\times$ F_1) and BC_2 (F_1 $\times$ Pusa Narangi Gaiinda). All six generations (P_1 , P_2 , F_1 , F_2 , BC_1 and BC_2) were evaluated to study the male sterile gene inheritance pattern. The progenies were phenotyped for male sterility based on floral characteristics. The segregation pattern of fertile and sterile plants was worked out by manual counting.

DNA isolation

The DNA was isolated from the young leaves of both the parents, and 96 samples of DNA were isolated from the F_1 population (petaloid sterile $\times$ PNG) by using the CTAB method as described by Doyle and Doyle (1990) with some minor modifications, including RNAase treatment. The quality was analyzed by using 0.8% agarose gel and quantity by using a UV-Vis spectrophotometer. The final DNA concentration was adjusted to 60 ng/ul.

Genotyping

The eleven SSR microsatellite markers linked to apetaloid male sterility viz CPSSR4, CPSSR7, CPSSR11, CPSSR16, CPSSR26, CPSSR33, CPSSR37, CPSSR39, CPSSR47, CPSSR53 and CPSSR66 shortlisted in the previous report (Asha *et al.*, 2019) were used in the present study (Table 4). The parental polymorphic SSR markers were utilized for screening the F_1 population to evaluate the co-segregating nature and confirm the linkage with sterile loci.

PCR analysis

Genotyping was carried out in an Eppendorf Thermocycler (Eppendorf master cycler Germany) with an initial denaturation at 94°C for 2 minutes, followed by 35 cycles; each cycle consisting of denaturation at 94°C for 45 seconds, primer annealing at 60°C for 45 seconds and primer extension at 72°C for 45 seconds and a final extension for 10 minutes at 72° C and hold at 12 °C. The 20 μ l reaction mixture contains 60 ng of template DNA, 0.2 X buffer, 0.2 mM dNTPs, 0.5 mM of MgCl₂, 0.3 U of Taq DNA polymerase, and 0.025 pM each of forward and reverse primers. The PCR product was separated by using 45 agarose or 8% PAGE. The results were documented by using the UV gel documentation unit. The allele sizes are calculated by using Uv-pro software.

Statistical analysis

The statistical analysis was performed using Statistical Analysis System Version 9.3 software (SAS, 2012). Phenotypic and genotypic data were analyzed by the Chi-square test to check the co-segregation and to determine the goodness-of-fit (Quinn and Keough 2002). Genotypic data were subjected to single marker-ANOVA to determine an independent assortment of markers with that of phenotype.

RESULTS

Identification of the stable genic male sterile lines

The intercrossing between fertile and sterile plants in all three petaloid lines (IIHRMOP 1111, IIHRMOP 22 and IIHRMOP 228), resulted in progenies with fertile and sterile plants of 22:21, 15:19, and 15:23 respectively with the segregation ratio of 1:1 while

selfing of fertile plants resulted in progenies consisting of all fertile plants (Table 1). Out of three lines, IHRMOP 1111 is selected based on the probability (87%) of the chi-square value for further analysis. Inter-cross and selfing performed over three different seasons in petaloid male sterile line IHRMOP 1111 confirmed the stable performance of the line. The segregating patterns across the seasons resulted in 27-23, 63-55, and 25-28 sterile and fertile plants segregating in a 1:1 ratio in intercrossing and selfing of fertile plants, resulted in progenies comprised of all fertile plants (Table 2). This clearly showed the male sterile line is stable across three generations.

Genetics of male sterility in petaloid male sterile system

In six generations out of 96 plants in F₁ resulting from IHRMOP 1111(P₁) crossed with Pusa Narangi Gaiinda (P₂), 40 were petaloid sterile, and 56 were fertile with the segregation ratio of 1:1. The fertile plants in the F₁ are selfed to produce the F₂. All 210 plants of F₂ were observed to be fertile, while the BC₁ 34 sterile and 24 fertile plants were observed, and BC₂ generation plants segregated into 21 sterile and 24 fertile plants in a ratio of 1:1 (Table 3).

Molecular markers for petaloid sterility

The parent IHRMOP 1111 and Pusa Narangi Gaiinda were assessed for polymorphism with eleven molecular markers earlier shortlisted (Table 4). Among the eleven markers assessed, CPSSR 39 was found to be polymorphic and could differentiate sterile and fertile genotypes.

Validation of CPSSR-39 association with sterility trait

The PCR amplification using CPSSR 39 marker in petaloid sterile line resulted in two amplicons with size 350 bp and 290 bp and a single amplicon with size 280 bp in fertile pollen parent Pusa Narangi Gaiinda. This showed a clear polymorphism between the two parents. Genotyping of 96 individual population of the F₁ population using CPSSR 39 resulted in a heterozygous banding pattern in 40 sterile individuals and a homozygous banding pattern in 56 fertile individuals with the same amplification PCR products sizes as of parents, co-segregating in accordance with the phenotype of each individual as per expectation; differentiating sterile and fertile individuals in 1:1 ratio (χ^2 $p < 0.05$, 2.66) (Figure 2(i, ii, iii)). The segregation of the polymorphism fitted into the expected 1:1 ratio with a probability of 0.10% confirms the linkage of these markers to petaloid male sterility. The single marker-ANOVA revealed the association between SSR marker with sterility in a segregated F₁ population of marigold at a 1% level of significance with an F value of 133. Since CPSSR 39 followed the mendelian segregation ratio, we presume CPSSR 39 is linked to male sterility loci in the petaloid genotype. Hence this marker CPSSR 39 can be efficiently used for the selection of parents in marker-assisted breeding programs.

DISCUSSION

Diversification of male sterility sources in hybrid seed production is essential to avoid the possibility of disease and pest attack and to reduce the impact of loss (Levings 1990). Easy maintenance of male sterility is another issue of concern, as vegetative propagation reported in marigold has its own limitations (Kumar et al., 2017; Tejaswini et al., 2016a). In this study, work was carried out using a genic petaloid male sterile line to understand the inheritance pattern of genes controlling it.

Based on the segregation pattern observed (Table 1, Table 2), the segregating progeny of 1:1 fertile and sterile plants observed under intercross and all fertile plants resulting from the selfed progeny of fertile plants explains petaloid sterile plants being heterozygous and fertile plants of the sterile line being homozygous recessive in nature. Hence we can assume that the male type of male sterility in the lines is GMS and governed by a single dominant gene Pp for petaloid sterility. Assuming the heterozygous status of petaloid male sterility as confirmed from the first experiment, line IHRMOP 1111 is expected to have male sterile plants with 'Pp' and fertile plants with pp genetic composition. Pusa Narangi Gaiinda, a well-established variety with all fertile flowers, is expected to be of 'pp'. The observed phenotypic data over the six generations consistently fitted to the Chi-square ratios as per the expectations is with in accordance with the assumption. All the results across the season and across multiple generations confirmed the existence of a single dominant gene governing petaloid male sterility. The PCR amplification patterns are with respect to the Pp and pp confirming the gene action at the genotypic level. Hence we propose a single dominant gene controlling petaloid male sterility and CPSSR 39 is linked to male sterility loci in petaloid genotype.

Irrespective of crops, most of the reported male sterility was either monogenic recessive or dominant (Reddy et al., 2002; Joshi and Nabi, 2018; Hundal and Khurana, 2001). In marigold, apetaloid sterility was reported to be controlled by a single recessive gene (He et

al., 2009; Tejaswini et al., 2016b). In contrast to apetaloid male sterility, it is now confirmed the presence a single dominant gene governing petaloid male sterility in IHRMOP 1111 genotype. With this present study, the presence of different genes responsible for different types of male sterility in marigold is confirmed. More than one gene is known to exist governing male sterility in different crops. For instance, in the case of pigeon pea, ms1 and ms2 genes were non-allelic and monogenic recessive reported to be governing male sterility (Saxena et al., 2010).

The most important aspect of the identified male sterile line in the present work is the easily distinguishable character of male sterile plants wherein flowers consist of only ray florets, unlike the presence of disc florets in fertile flowers (Fig. 1). In general, male sterile plants are morphologically not distinguishable from the sister fertile plants, except in a few cases where the male sterile flower size is smaller than that of fertile flowers, e.g. tomato, chilli (Sawhney 1983).

Classical A, B, C, D, and E model explains the genetic regulation of floral organ development. Accordingly, Class B genes in combination with A govern petal formation, while B and C group together govern stamen formation (Theissen 2001; Weigel and Meyerowitz 1994). Homeotic mutation in these genes explains any abnormalities seen in floral organs, including male sterility due to the absence of stamens. Petaloid sterile flowers studied in the present work indicate the possible conversion of stamens into petals. In carrots, it has been reported that the anthers of individual florets are replaced by petal-like structures that don't release pollen, resulting in petaloid male sterility (Morelock et al., 1996). Roses of double-flowered forms with many extra petals display a significant contraction in the domain of C gene expression, allowing for a much larger domain of A + B expression and concomitant extra petals (Bowman et al., 2012). Similarly, petaloid marigold types may be a result of similar ABC expression as of double-flowered roses.

Homeotic conversion of ray and disc florets into sepal and style-like structures corresponding to abnormality of androecium and differential expression of B-class genes in floral development have been reported in apetaloid male sterile flowers of *T. erecta* (Ai et al., 2016; He et al., 2010). The petaloid sterile line reported announces that *T. erecta* is an ideal material for the study of homeotic genes as well as serving as an important breeding material for hybrid seed production.

The results suggested the CPSSR 39 marker linked to petaloid sterility as a selection marker in MAS breeding that facilitates the identification of petaloid sterile types enabling the removal of fertile plants at the seedling stage, retaining only sterile progenies during the hybridization programme in marigold. The markers identified to be linked to apetaloid male sterility in marigold using SCAR marker SCS48, reported by He et al., 2009; Asha et al., 2019 validated the same SCAR marker in 12 apetaloid male-sterile lines and confirmed the efficiency of the MAS. Similarly, markers linked to male sterility in various crops like sunflower (Pérez-Vich et al., 2005), rape seed (Lu et al., 2004, Lee et al 2010, and Hong et al., 2008), Chinese cabbage by Ying et al., 2003 and Hui et al., (2011), Carnation by Yagi et al., (2014), rice by Bhati et al., (2018) and Raghavendra and Hittalmani (2015), tomato by Kumar et al., (2018), chilli by Aulakh et al., (2017), Onion by Dhanya et al., (2014), welsh onion by Gai *et al.*, (2010), Barley by Emebiri (2010) was reported which explains the significance of markers in breeding of male sterile hybrids.

CONCLUSION

The present study confirmed petaloid male sterility in the marigold is governed by a single dominant gene based on its inheritance pattern. Apetaloid and petaloid male sterility are the two different types of male sterility in marigold based on flower structure and the present study suggests the existence of two different genes responsible for the expression of structurally variable male sterility in marigold. This study also indicated the possibility of considering marigold (*T. erecta*) as a candidate species for the study of the homeotic conversion of floral structure.

Identification of marker CPSSR 39 associated with petaloid male sterility has the potential application in the marker-assisted selection breeding programmes in marigold. MAS enable accurate selection regardless of environmental factors, and it has been applied in the breeding of various crops, but to the best of our survey, the development of a commercial marigold cultivar through MAS has not been reported. Male sterility linked markers in the present study indicate the possibility of MAS and the potential use of CPSSR 39 in the hybrid breeding programme of marigold. In conclusion, once molecular markers have been linked to a trait of interest, these markers can be used to select desired plants from a large population through marker-assisted selection (MAS), which saves both costs and time in hybrid breeding programmes.

Declarations

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Tables

Table 1. Segregation pattern observed in intercross and selfed progeny of stabilised petaloid male sterile lines.

S.No	Intercross and selfing of petaloid sterile lines	Number of sterile plants observed	Number of fertile plants observed	Expected ratio of sterile to fertile plants	Chi-square value	Probability
1	IIHRMOP 1111					
a	IIHRMOP 1111-s X IIHRMOP 1111-f	22	21	1:1	0.02	0.87
b	Self of IIHRMOP 1111-f	-	25	0:1	-	
2	IIHRMOP 22					
a	IIHRMOP 22-s X IIHRMOP 22-f	15	19	1:1	0.47	0.49
b	Self of IIHRMOP 22-f	-	8	0:1	-	
3	IIHRMOP 228					
a	IIHRMOP 228-s X IIHRMOP 228-f	15	23	1:1	1.68	0.19
b	Self of IIHRMOP 228-f	-	5	0:1	-	

* s-sterile; f-fertile

Table 2. Segregation pattern observed in intercross and selfed population of petaloid male sterile line IIHRMOP 1111 (IIHRMOP 1111-s + IIHRMOP 1111-f) in different seasons.

S.NO	Intercross and selfing of petaloid male sterile lines	Number of sterile plants observed	Number of fertile plants observed	Expected chi-square ratio	Chi-square value	Probability
Season-1						
a	IIHRMOP1111-s X IIHRMOP 1111-f	27	23	1:1	0.32	0.57
b	Self of IIHRMOP 1111-f	-	35	0:1	-	
Season-2						
a	IIHRMOP1111-s X IIHRMOP 1111-f	63	55	1:1	0.54	0.46
b	Self of IIHRMOP 1111-f	-	125	0:1	-	
Season-3						
a	IIHRMOP 1111-s X IIHRMOP 1111-f	25	28	1:1	0.16	0.68
b	Self of IIHRMOP 1111-f	-	45	0:1	-	

* s-sterile; f-fertile

Table 3. Segregation pattern observed in six-generations of crossing program between IIHRMOP 1111 and Pusa Narangi Gaiinda for confirmation of genes involved in petaloid male sterility.

S.No	Initial material maintained/crossed		Resulting progeny					Chi-square value p=0.05	Probability
	Lines/varieties used in crossing	Genotype (Sterile: Fertile)	Generation	Expected phenotypic ratio (sterile:fertile)	Expected genotypic ratio (sterile:fertile)	Number of sterile plants- observed	Number of fertile plants- observed		
1	IIHRMOP 1111 (IIHRMOP 1111-s + IIHRMOP 1111-f)	1Pp:1pp	P ₁	1:1	1Pp:1pp	25	28	0.16	0.68
2	Pusa Narangi Gaiinda (PNG)	0:pp	P ₂	0:1	0:pp	0	45	-	-
3	IIHRMOP 1111-s X PNG	Pp x pp	F ₁	1:1	1Pp:1pp	40	56	2.66	0.10
4	Self of fertile F ₁	pp x pp	F ₂	1:0	pp:0	0	210	-	-
5	IIHRMOP 1111-s X Fertile F ₁	Pp x pp	BC ₁	1:1	1Pp:1pp	34	24	1.72	0.18
6	Sterile F ₁ X PNG	Pp x pp	BC ₂	1:1	1Pp:1pp	21	24	0.2	0.65

*s-sterile; f-fertile

Table. 4. The sequences of markers used in the present study are as follows:

Sl. No	Markers name	Primer sequence (5' – 3')	
		Forward primer	Reverse primer
1	CPSSR 4	TCCACATCAAATTCTTGGTCCCT	GGGGAGGGTCGTTGCATATT
2	CPSSR 7	CGTGATGTGCGAACGTTGTGG	TGAAGGTGGTGGTGCCTTTT
3	CPSSR 11	AGAGAGAGAGACCACTGTTGT	TCACAACATCACAGCTCAACAC
4	CPSSR 16	CCATTAAAGGGCTCGACGGA	GGACTTGCTCCGCTACCTAC
5	CPSSR 26	GCTGTTGGAGCCACTGATCT	ACATCAATCCCTACAAAACCCT
6	CPSSR 33	CAATTTTCGTTCCGGCTGCA	GAGCATGTTGCCTCAGAGGT
7	CPSSR 37	ACCCGTACCCAATCCCAATT	GCAGCACTACTACAACCACCA
8	CPSSR 39	ACTCACGGGAGGAGAAATGC	CAGAAGCAGAGACCGGTCTG
9	CPSSR 47	TCGGGGAGATGTCTGAATTTGG	CGTCACGCATAAACGAATGT
10	CPSSR 53	TGGGATGATCTGGGAGCTGA	AGTGTCCAACCAAAAGCCCTA
11	CPSSR 66	CGATGACGTTGACGGACTTTG	AGGCCGAATTGAAGGTGATGT

Figures

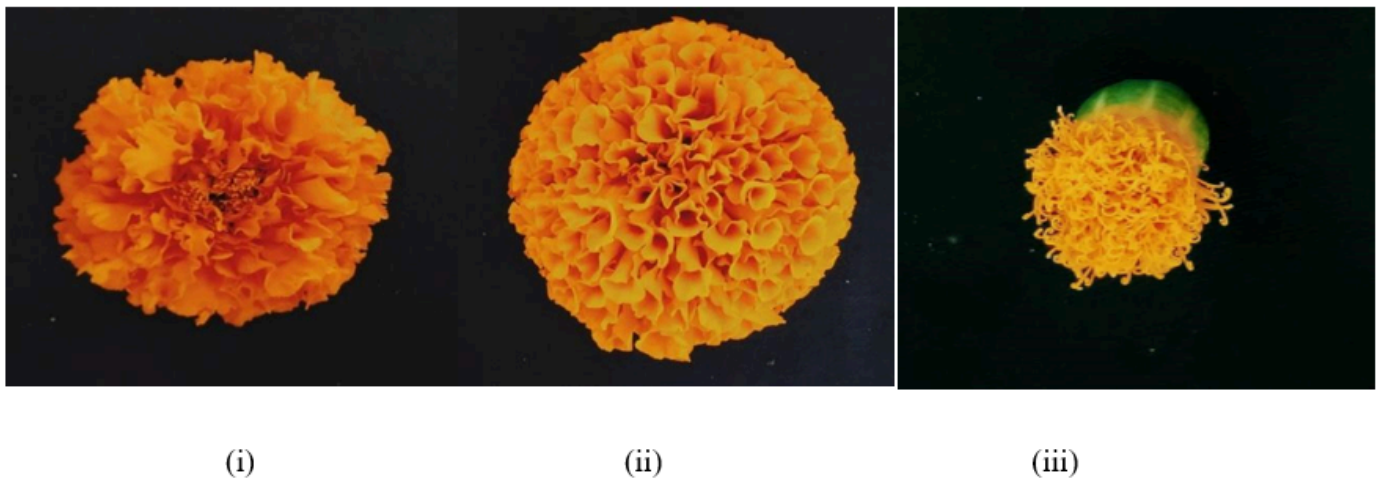


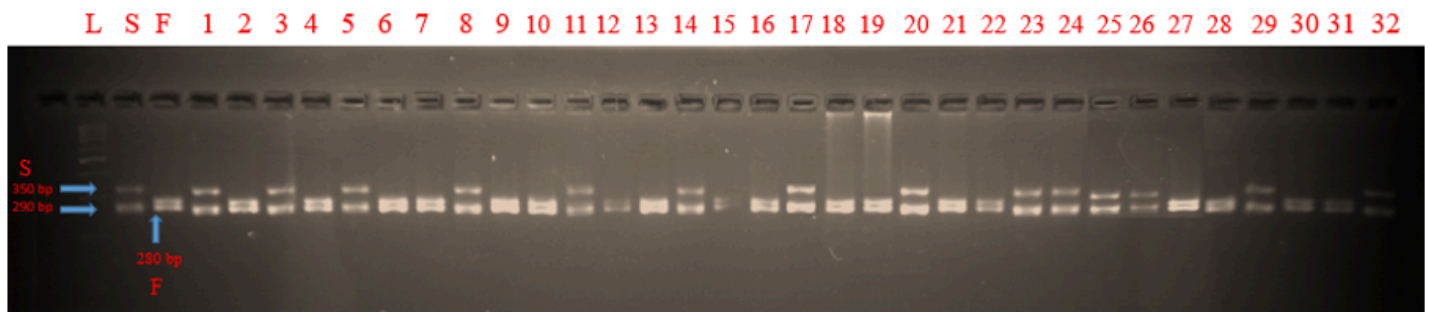
Figure 1

Petaloid sterile flower with only ray florets, apetaloid sterile flower with only gynoecium, fertile flower with both ray and disc florets.

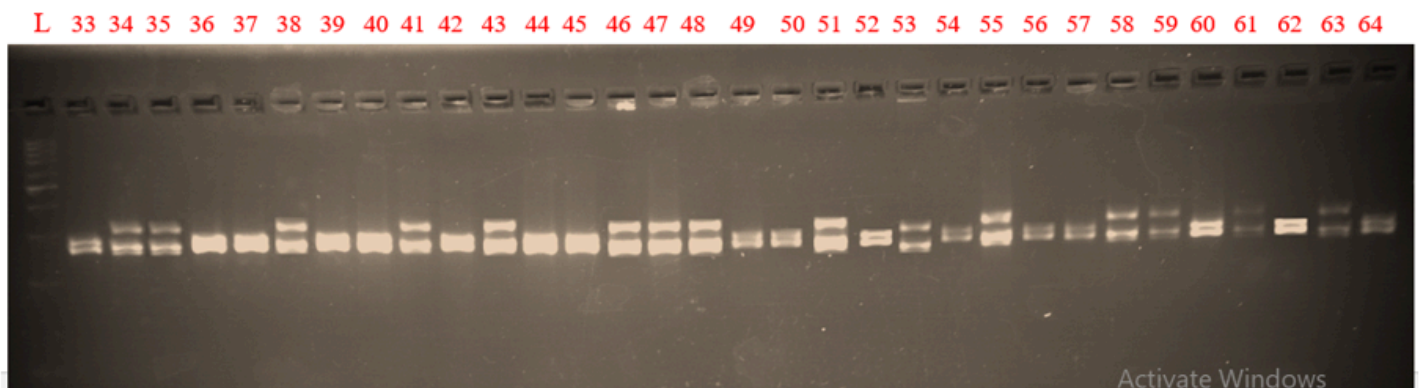
(i) Male Parent (Pusa Narangi Gaiinda)

(ii) IIHRMOP 1111 (Petaloid sterile)

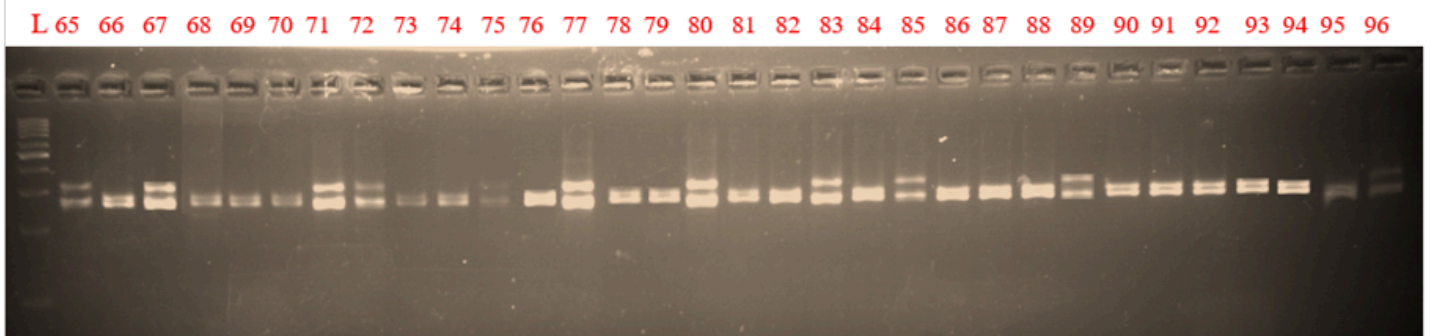
(iii) (Apetaloid sterile)



(i)



(ii)



(iii)

Figure 2

The PCR amplification of CPSSR 39 with sterile, fertile parents and F_1 population of 1-96 individuals; the sterile parent with heterozygous amplified at 350 and 290 bp; fertile parent at 280 bp. The population co-segregating with in accordance to parents with 40 sterile and 56 fertile individuals in 1:1 ratio.

(i) The PCR amplification of CPSSR 39 for petaloid F_1 population with sterile, fertile parent and 1-32 individuals.

(ii) The PCR amplification of CPSSR 39 for petaloid F_1 population 33-64 individuals.

(iii) The PCR amplification of CPSSR 39 for petaloid F_1 population 65-96 individuals