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Comparative Evaluation of Traditional Grow-Out Test and Molecular Finger-Printing for Testing of Hybrid Purity in Pumpkin (*Cucurbita moschata* L.)

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ABSTRACT

The primary method for hybrid purity analysis is to use morphological distinctions between true to types and off types in grow out tests (GOTs). Traditional GOT was costly, time consuming, and environmentally sensitive. Recent developments in DNA markers show promise for speeding up and improving the accuracy of hybrid genetic purity testing. The purity of pumpkin hybrid PPH1 was evaluated in this study using both standard GOT and sophisticated molecular marker techniques. The experiment involved mixing 95% F_1 hybrids with 5% female parents in individual sample sets of 300, 200, 100, 50, and 25. To ensure hybrid purity, a PCR based assay and GOT were performed on each sample size. The PCR based technique examined 37 pairs of SSR markers, two of which showed parental polymorphism. The level of purity had assessed using co-dominant marker. The smaller sample size of 100 had been analysed to validate hybrid purity, and results were consistent with those of larger sample sizes (300, 200). As a result, it was claimed that molecular marker-based hybrid purity evaluation could be a viable alternative to classical GOT.

Keywords: Assay, Grow-out test, Hybrid, Pumpkin, Purity.

INTRODUCTION

Seed is a critical and essential input in agricultural production and seed quality contributing approximately 20 to 25 percent to overall productivity. Therefore, ensuring the availability of high-quality seeds to farmers across the country (Anonymous, 2025). To achieve higher yield and reap maximum profits from vegetable cultivation, the farmers are showing ample interest in hybrid seeds of vegetable crops. To cater increasing demand of high-quality seeds, numerous hybrids of various vegetable crops have been released by public as well as private sector. The private companies are luring the farmers with lucrative advertisements and marketing gimmicks. To make a quick buck, some of the private companies are supplying spurious hybrid seeds of poor quality to the farmers, which more often results in losses rather than profits. Therefore, for successful commercialization of hybrids, it is mandatory to examine the genetic purity, to assure uniformity and stability of field performance and yield. The prime objective always is to maintain purity at the genetic level, which ultimately helps to exploit the full potential of the hybrids. When the seeds are passed from one generation to another, genetic contamination may occur and if it is unnoticed in the population it may lead to genetic deterioration of the variety (Yalamalle *et al,* 2008). Therefore, a reliable method for distinguishing between cultivars and assessing the genetic purity of seed samples is essential. Such a method would enable seed producers to monitor and maintain the required levels of genetic purity throughout each generation of seed production and multiplication, ultimately ensuring the delivery of high-quality seeds (Kiruthika and Padmanabha, 2018). The most crucial factor in determining the quality of seed is its hybrid purity, which is tested to determine any deviations from the variety's authenticity (Chesnokov, 2005). A high level of genetic purity assessment must be maintained in order to certify seeds and market hybrids.

The fundamental technique for analysing hybrid purity uses the morphological variations among true-to-types as well as off-types in the grow out test (GOT) (Shinde *et al,* 2021). Traditional GOT are often cost-intensive, laborious, time-consuming and highly sensitive to environmental conditions. The detection of morphological traits at different plant growth stages is the foundation of the traditional grow-out test (GOT)

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method for determining genetic purity (Alam *et al,* 2015) but this method is time-consuming and susceptible to external influences. Due to limited polymorphism and environmental effects, biochemical techniques like isozyme analysis and seed protein electrophoresis are unable to distinguish between genotypes that are closely related. Additionally, they do not provide exact figures for genetic distances between cultivars. Furthermore, there is a rising need for a unique hybrid purity testing method that could produce reliable, timely, affordable results (Pattanaik *et al,* 2018).

Since DNA based markers are based on the genotype of the hybrids, which eliminates environmental changes, they can be used to identify purity of hybrid at seed level, overcoming limitations of morphological or biochemical markers. The co-dominant, highly informative, repeatable, and most reliable indicators for determining hybrid purity are simple sequence repeats (SSRs) (Zhao *et al,* 2017). The development and successful use of molecular markers in numerous elements of crop improvement programs have occurred recently. In comparison to morphological and biochemical markers, which have a limited quantity and a slow rate of data collection, molecular markers provide a number of advantages (Daniel *et al,* 2012). They are regarded as a useful tool since they may identify genetic variants and are unaffected by environmental factors (Tanksley and McCouch, 1997). The molecular markers have been used to test the purity of seeds in numerous studies (Powell *et al,* 1996; Ajmone *et al,* 1998; Bornet and Branchard 2001; Mammadov *et al,* 2010). These markers are quicker to assay, use a less amount of DNA template, and take less time. But their biggest drawback is that they are harder to duplicate. The most often employed hybridization-based molecular marker for identifying DNA sequence polymorphism is called a restriction fragment length polymorphism (RFLP). They are co-dominant and highly reproducible, but their use is constrained by their time-consuming nature and high demand for high-quality DNA (Kesawat and Das, 2009). In the present study, the PCR based DNA marker system of microsatellite (SSR) was employed for identifying two commercial hybrids of pumpkin released by Punjab Agricultural University, Ludhiana and their respective parents for their utility in determining the genetic purity. These markers were also validated for the purpose of testing the genetic purity of the commercial F_1 seed lots to establish an efficient, reliable and accurate method for rapid testing of genetic purity of commercial hybrids when compared to the traditional grow out test.

MATERIALS AND METHODS

Plant material

The materials utilized in this study consisted of Pumpkin F_1 hybrid PPH1 along with its parental lines P112 and BN364 developed by Punjab Agricultural University Ludhiana, Punjab, India. The differential morphological characters from the hybrids PPH1 and its parental lines were used to identify the selfed/off-types during grow out test.

GOT analysis

To assess conformity between molecular marker-based estimates of selfed/off-type plants through phenotypic observations, experiment had been conducted by pooling 95% F_1 hybrids merged with 5% female parents, individually sets of samples *viz,* 300, 200, 100, 50, and 25. All five sample sizes, as well as the parental lines PPH1, were seeded in the tray for 15-30 days before being transported to the main field for GOT analysis. Throughout the plant's growth, measures such as correct irrigation, fertilizer, and insect management were implemented. Purity evaluations were conducted using essential morphological parameters mentioned in DUS (distinctness, uniformity, and stability) guidelines, such as plant habit, leaf shape, color of leaf, fruit shape, fruit main skin color at mature and immature stages throughout the growing season (Table 3). To ensure hybrid purity, the PCR-based test and GOT were performed on each sample size. The selfed/off type plants found using SSR marker research had been confirmed using morphological characteristics. The experiment was replicated three times, the mean percentage of hybrid purity, had been calculated and determined using both PCR-based assay and GOT.

Hybrid purity (%) = Total number of plants number of off-types/ Total number of plants× 100.

Isolation and quantification of DNA

Genomic DNA had been isolated from the leaves of parental lines along with their hybrids across all sample sizes *i.e.,* 300, 200, 100, 50, 25 individuals using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol. The quantity as well as quality of the extracted genomic DNA had assessed through electrophoretically resolved 2.5 percent (w/v) agarose gel stained with good view dye, amplified PCR products were separated.

Table 1. Time taken to determine the purity of Pumpkin hybrid PPH1 by GOT and molecular-based assay

	GOT	Molecular based assay
Duration	Around 70 Days	48 h (after seedling stage)

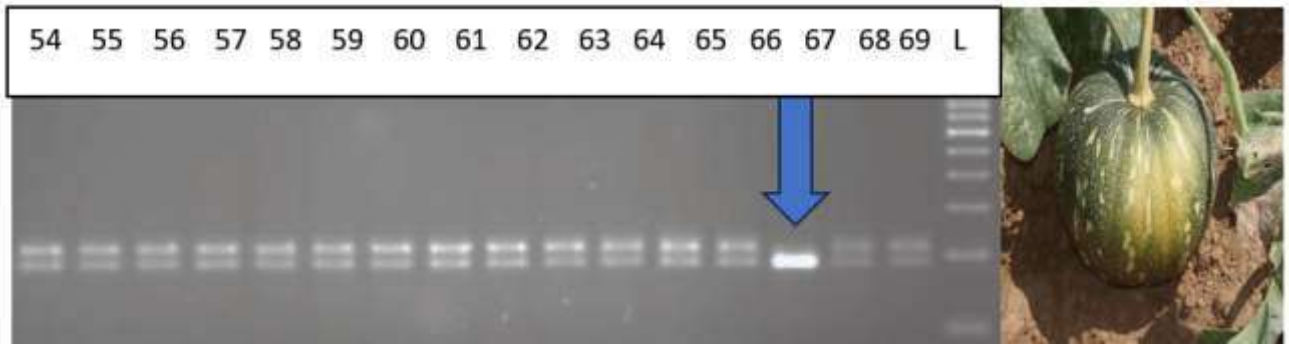
Table 2: Morphological characters used to identify selfed or off types during GOT for PPH1 and its parents

Sr. No.	Morphological character	Female parent of PPH1 (P112)	Male parent of PPH1 (BN364)	PPH1
1.	Stem	Sulcate	Sulcate	Rigid
2.	Peduncle of fruits	Angulate with obtuse ribs	Angulate with obtuse ribs	Rigid
3.	Peduncle length	Long (>10cm)	Long (>10cm)	Short (<5cm)
4.	Fruit: main colour of the skin (at immature fruit stage)	Dark green	Medium green	Light green
5.	Fruit: skin colour pattern	Mottled	Mottled	Uniform
6.	Fruit shape at peduncle end	Raised	Raised	Moderately depressed
7.	Fruit: marbling (immature stage)	Medium	Long	Medium
8.	Fruit shape	Oval	Oval spherical	Oval
9.	Fruit waxiness of skin (at mature stage)	Light	High	Medium
10.	Flesh thickness	Thick (>4.5cm)	Medium (2.5-4.5cm)	Thick (>4.5cm)

Fig. 1: The variability in morphological characters of pumpkin hybrid PPH1 along with its parents. (a) plant habit and leaf shape (b) Fruit shape and colour

Polymorphism analysis by SSR marker

The overall 37 pairs of SSR markers had been utilized to investigate polymorphism in bulk of DNA collected from 10 seedlings of male as well as female parent of the Pumpkin PPH1 hybrid. Polymorphic SSRs had been selected for hybrid purity testing of PPH1. DNA amplification was performed in a 20 µl mixture of reaction with 5 pmol primers, 10X Taq polymerase buffer, 1 mM dNTP, 20 ng template genomic DNA and 3U of Taq polymerase. The amplification had initiated with a denaturation step at 94°C for 3 minutes, afterwards 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute with a final extension at 72°C for 10 minutes. Amplified DNA fragments had separated on a 3% (w/v) agarose gel with 0.5 µg/ml ethidium bromide in 45 mM Tris borate (1 mM) EDTA buffer, pH 8.0. The well was loaded with 20 µl of PCR products and 5 µl of loading solution. The molecular standard was a 100-base pair (bp) DNA ladder. Electrophoresis was performed at 70V for 3 hours as well as gels were seen under UV transilluminator through the UV Pro gel documentation system.



a.Hybrid PPH1 comparison of GOT and SSR analysis

Fig. 2: Phenotypically similar F_1 hybrid PPH1 showing genotypically off-types/selfed

Table 3: Comparison of hybrid purity assessment based on morphological and molecular markers across varying different sample sizes in pumpkin hybrid PPH1 and parents

Sample size	Replicate 1				Replicate 2				Replicate 3				Mean GOT assay	Mean SSR assay
	GOT assay	Purity %	SSR analysis	Purity%	GOT assay	Purity %	SSR analysis	Purity%	GOT assay	Purity %	SSR analysis	Purity%		
Sample size 300 mixed with 5% admixture expected Observed no. of true-to-type hybrids Observed no. of off types/selfed	285 283 2	99.3	285 283 2	99.3	285 281 4	98.6	85 281 4	98.59	285 285 0	100	285 284 1	99.7	283 (98.9%)	282.67 (99.2%)
Sample size 300 mixed with 5% admixture expected Observed no. of true-to-type hybrids Observed no. of off types/selfed	190 189 1	99.5	190 189 1	99.5	190 190 0	100	190 190 0	100	190 187 3	98.4	190 187 3	98.4	188.7 (99.3%)	188.7 (99.3%)
Sample size 300 mixed with 5% admixture expected Observed no. of true-to-type hybrids Observed no. of off types/selfed	95 95 0	100	95 95 0	100	95 94 1	98.9	95 94 1	98.9	95 95 0	100	95 95 0	100	94.7 (99.7%)	94.7 (99.7%)
Sample size 300 mixed with 5% admixture expected Observed no. of true-to-type hybrids Observed no. of off types/selfed	46 45 1	97.8	46 46 0	100	46 43 3	93.48	46 43 3	93.48	46 46 0	100	46 46 0	100	44.7 (99.3%)	45 (98%)
Sample size 300 mixed with 5% admixture expected Observed no. of true-to-type hybrids Observed no. of off types/selfed	23 23 0	95.65	23 23 0	100	23 22 1	95.65	23 22 1	95.65	23 23 0	100	23 23 0	100	22.7 (98.7%)	22.7 (98.7%)

RESULTS AND DISCUSSION

Genetic purity assessment using GOT and SSR markers

In this study, plants from five different sample sizes (300, 200, 100, 50 and 25) of PPH1 each plant was individually evaluated to determine its true-to-type status based on ten morphological characteristics in the GOT. Female parent P112 can be distinguished from male parent BN364 and hybrid PPH1 on the basis of following characters: Stem, peduncle of fruits, length of peduncle, fruit: main colour of the skin, fruit shape at peduncle end, fruit: skin colour pattern, fruit: marbling, shape of fruit, fruit waxiness of skin, flesh thickness. The stem of the male parent BN364 as well as female parent P112 was sulcate, whereas the hybrid PPH1 exhibited a rigid stem. The above results were closely associated with previous findings of Gediya *et al* (2018). The fruit peduncle of male parent BN364 and its female parent P112 had an angulate shape with obtuse ribs, whereas the hybrid P112 demonstrate a rigid structure. The similar findings were in accordance with Vincent *et al* (2022) who studied the morphological traits of pumpkin. The peduncle length of the male parent BN364 and the female parent P112 was long (>10 cm), while that of the hybrid PPH1 was short (<5 cm). The similar findings had also been documented by Nagar *et al* (2017). At the immature fruit stage, the fruit: main colour of the male parent BN364 was medium green, the female parent P112 indicates a dark green skin, while the hybrid PPH1

showed a light green colouration. The above results stood parallel with the findings of Tabasum *et al* (2021). The fruit: skin colour pattern of both the male parent BN364 and the female parent P112 was mottled, whereas the hybrid PPH1 demonstrated a uniform pattern and fruit shape at the peduncle end was raised in both the male parent BN364 and the female parent P112, while the hybrid PPH1 indicates a moderately depressed peduncle end. These results were in accordance with Chaudhary *et al* (2015). At the immature stage, the fruit: marbling of the male parent BN364 and the hybrid PPH1 was medium, whereas the female parent P112 exhibited long marbling fruit. The above results were closely associated with previous findings of Ramjan *et al* (2018) who observed the morphological parameters of pumpkin genotypes. The fruit shape of the male parent BN364 was oval-spherical, while both the female parent P112 along with hybrid demonstrated an oval shape. The similar findings were in accordance with Kiramana *et al* (2017) who studied the conventional pumpkin landraces can supply the genetic material needed to improve crops. The fruit skin waxiness of the male parent BN364 was high, while that of the female parent P112 was low, hybrid PPH1 indicates a medium level of waxiness. These results were in accordance with Chen *et al* (2020). The flesh thickness of the male parent BN364 was medium (2.5–4.5 cm), whereas both the female parent P112 and the hybrid PPH1 exhibited thick flesh (>4.5 cm). The above results stood parallel with the findings of Ruelas *et al* (2015).

SSRs were the most commonly used and reliable DNA-based markers for assessing genetic purity. A single co-dominant marker proved effective in identifying off-type hybrids during the purity assessment of the pumpkin hybrid PPH1 using SSR molecular analysis. The total of 37 SSR primers had been screened to determine hybrid purity of pumpkin hybrid PPH1. Out of 37 primers screened, only one primer *viz.,* comp28857 was found to be polymorphic which had potential to identify hybrid purity of pumpkin. The above results were closely associated with previous findings of Miladinović *et al* (2016). The average hybrid purity percentage determined by GOT assay for sample set of 25, 50, 100, 200 and 300 sample sizes of PPH1 was calculated to be 98.7, 98.0, 99.7, 99.3, 99.2 respectively (Table 3).

Duration involved in determining the purity of Pumpkin hybrid

GOT, which assesses plant morphology, took approximately 70 days to identify selfed or off kinds in 6 sample sizes. The PCR based assay required about 48 hours to determine admixtures, making it time saving method to assess purity of hybrid against selfed and off-type plants (Table 1). The present study implicated that the development of DNA markers might help to test cultivars purity developed from closely related parental lines. To ensure commercial success, the hybrid should be pure. Genetically pure hybrids function best when consistent, else they may perform poorly. Molecular markers must be used to ensure the genetic purity of hybrids created. Traditionally, GOT was used for testing the genetic purity, it takes the full season. Additionally, this method had been labour-intensive and susceptible to environmental changes, making it less reliable for assessing genetic purity. Hybrid seeds were unavailable for immediate growing, resulting in additional storage costs and an overall increase in hybrid seed prices. This work uses molecular markers to evaluate hybrid purity and provide farmers with true-to-type seeds. GOT tested genetic purity with a minimum sample size of 400 seedlings. However, maintaining such a large sample size proved challenging as well as labour intensive. As determined through testing six different sample sizes: 300, 200, 100, 50 and 25 seedlings were adequate to confirm the hybrid purity of pumpkin, with findings equivalent to larger sample sizes. The above results were closely associated with previous findings of Ana *et al* (2017). Marker analysis provided consistent results with GOT. GOT was a time-consuming and labour-intensive method for determining genetic purity, as several distinguishing characteristics arise only during the blooming and post-flowering stages, taking roughly 70 days. As a result, it frequently causes delays in the entire decision making, packaging as well as marketing of commercial seeds. As a result, farmers don't receive hybrid seeds at time of sowing, prevent them from being planted straight away. Hybrid seed prices rise due to increased investment in production along with storage. The limitations of GOT could be effectively overcome through the use of molecular markers. These results were in accordance with Pattanaik *et al* (2018). SSR analysis might performed in laboratory, which takes 48 hours to evaluate genetic purity, reducing labour, space and time spent on purity testing. Using locus-specific allelic information from SSR markers allows for more precise and efficient differentiation of pumpkin hybrids from parental lines and off-types/selfed varieties. These results were in accordance with Adeyemo *et al* (2019). The variety or hybrid genetic purity was examined by SSR markers along with GOT shows similar results, indicating that molecular markers can support GOT testing. Implementing marker analysis was cost-effective and

would benefit the seed industry. The above results stood parallel with the findings of Kaur *et al* (2023). Additionally, commercially available genetic SSR markers might be used to assess the genetic purity of cultivars. The SSR marker data generated in this study can assist the seed industry in selecting optimal marker combinations for accurate assessment of crop genetic purity.

CONCLUSION

The study revealed that traditional GOT was costly, time consuming, and environmentally sensitive. Recent developments in DNA markers show promise for speeding up and improving the accuracy of hybrid genetic purity testing. The purity of pumpkin hybrid PPH1 was evaluated in this study using both standard GOT and sophisticated molecular marker techniques. The experiment involved mixing 95% F_1 hybrids with 5% female parents in individual sample sets of 300, 200, 100, 50, and 25. To ensure hybrid purity, a PCR based assay and GOT were performed on each sample size. The PCR based technique examined 37 pairs of SSR markers, two of which showed parental polymorphism. The smaller sample size of 100 had been analysed to validate hybrid purity, and results were consistent with those of larger sample sizes (300, 200). As a result, it was claimed that molecular marker-based hybrid purity evaluation could be a viable alternative to classical GOT.

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