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Identification of off-type in F1 Hybrids of Commercially Cultivated Vegetable Crops using SSR Markers as a Molecular Tool

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Abstract: ***The undesirable traits generally referred as "Off-type", in F1 generations is unacceptable beyond certain percentage for commercially cultivated crops. The traditional method of Grow-Out-Test is time consuming and laborious. Identification of the off-types with a Simple and powerful molecular marker tool before marketing is the need of the hour. In this present study the off-types are identified using SSR markers for Chilli, Tomato, Okra, cucumber and Bitter gourd. Collectively 96 crop specific SSR markers were screened for one variety from each crop and one polymorphic primer was identified for each parental line of all the crops. These polymorphic primers were validated on hybrids and the off-types were identified based on the banding pattern in comparison with the polymorphic allelic band of parents. These identified off-types were further allowed to grow completely till fruiting and the variation in fruit and leaves characteristic proved it be an off-types when compared with actual hybrid. So, SSR markers can be used as a quick and accurate means as a molecular marker tool for validating the commercially cultivated crops, which saves time and labor for commercial seed companies.***
Keywords: F1 Hybrids, Off-types, SSR Markers, Molecular tool

I. INTRODUCTION

"Off-type" are generally referred to any seed or plant that is not a part of the variety, in which it deviates in one or more characteristics from the variety that are described in control. These off-types are bound to occur when no proper controlled measures are taken during hybrid production and/or could be contaminated with other variety during storage and packing conditions. It becomes tough for seed companies to market if purity is not strictly maintained. Traditionally, Field Grow-Out-Test (GOT) was adopted for testing purity. But this is purely based on morphological markers that are time consuming, laborious and also influenced by environmental conditions (Cooke R.J., 1995). Alternatively, the emergence of Molecular makers has made the testing process easy, quick and accurate (Botstein et al., 1980) by determining genetic purity at lab level. In this process, the genuinity of the variety could also being authenticated if there is any deviation occurred during hybrid production. Genetic purity test is also compulsory for seed certification and marketing. Maintaining higher genetic purity is an essential prerequisite for the commercialization of any hybrid seeds. The seeds with low genetic purity could results in segregation of the traits, genetic deterioration of the variety and affect the yields in significant way. So, in order to overcome this disadvantage, and to speed up the testing procedures, DNA markers in addition to morphological markers can be used (Rakshit et al, 2008). Recently, several molecular markers such as RAPD, RFLP, AFLP, etc. (Maccaferri et al, 2007) were being used for screening most of the crops. However, repeatability and reliability is under question. But the use of co-dominant and extremely polymorphic molecular markers Such as simple sequence repeat (SSR) has given a new gateway in assessing the genetic purity more accurately in some of the crops like maize (Daniel et al., 2012), sunflower, and cauliflower (Zhao, et al., 2012). The SSR markers can be used for rapid assessment of genetic purity in hybrid and parental line seed purity (Iqbal et al., 2010, Pallavi et al., 2011, Chetan Kumar et al., 2012) and are of great importance in commercial application especially in assessing the parental line seed purity (Yashitola et al., 2002, Sundaram et al, 2008). The main objective of the present study was to identify the off-types in commercially cultivated Chilli Pepper (Capsicum annuum), Tomato (Solanum lycopersicum), Okra (Abelmoschus esculentus), Cucumber (Cucumis sativus) and Bitter gourd (Momordica charantia) hybrids together with their parental lines, using SSR markers as a tool.

II. MATERIALS AND METHODS

A. Plant Material

Five commercial crops, Chilli, Tomato, Okra, Cucumber and Bitter gourd hybrids were opted for this study. One variety from each crop was selected (Table I). 100 seeds from each variety were washed with water and treated with 0.5 % carbendazim 50 WP and

0.1% sodium hypochlorite for 5 minutes and washed with autoclaved water for 3 times. The seeds were germinated in sterile petriplates containing filter paper and incubated under artificial light for the duration as mentioned in Table I. The plants were irrigated with autoclaved water regularly. Approximately, 2mm^2 Leaf was harvested into a sterile 1.7 ml appendorf tubes and stored under -20^{0}C temperature until the extraction of genomic DNA.

TABLE I
Details of the plant materials used in this study and the duration of germination for each crop.

Crop	Variety	Parental Lines		Days for Germination
		Male	Female	
Chilli Pepper	HyChi - 01	CAM-01	CAF-01	10 - 15
Tomato	HyTom – 02	LEM-02	LEF-02	7 - 10
Okra	HyOk – 03	AEM-03	AEF-03	15 - 20
Cucumber	HyCum – 04	CSM -04	CSF- 04	15 - 20
Bitter Gourd	HyBit - 05	MCM-05	MCF-05	15 - 20

B. Molecular analysis

Plant DNA was isolated based on Hong Wang et.al, 1993. The harvested leaves samples retrieved from -20^{0}C temperature were thawed to room temperature. 25uL of 0.5 N NaOH is added in each 1.7 ml tube and the leaf tissues were crushed with plastic hand Crushers until there is no visible pieces of tissue are left. 5 µL from this was quickly transferred to a new tubes containing 495 µL of 100 mM Tris pH 8.0 and mixed well. 2 µL from this mixture was used for PCR amplification.

C. SSR Marker Polymorphic primer screening

Screening for Polymorphic primers for parental lines of each variety was studied. A total of 96 SSR markers obtained from the Solanaceae genome network and Cucurbit genomics data base were subjected for screening, to obtain a single and distinct polymorphic primer for each variety of the crops. The DNA extracted from Male and Female lines were subjected for Primer Screening. The stringency of the PCR conditions was varied to maximum to overrule the possibility of non specific amplification. The polymorphic primers were selected, PCR conditions were optimized and the DNA of 93 plants from each variety of a crop was subjected for PCR amplification along with their respective Parental lines. A negative control with no DNA was used during PCR amplification.

D. PCR Amplification

The PCR was performed in a total reaction volume mixture of 20 µL containing of 2 µL of DNA from crude lysate, 10 µL of Taq DNA Polymerase 2x Master Mix RED, 1 µL of 10 pico mole of each Primer and remaining volume was made up with 6 µL of autoclaved HPLC grade Water.

The PCR was carried out in a Thermal Cycler (Applied Biosystems) programmed for initial denaturation of 95^{0}C for 5 min, followed by 35 cycles of 94^{0}C (1 min), 56^{0}C (30 Sec.), 72^{0}C (1 min) then final-extension at 72^{0}C for 5 min. A volume of 10 uL of PCR product was used for electrophoresis. The amplicons were resolved on 3.5 % agarose gel stained with ethidium bromide and visualized under UV in a gel documentation system and image was captured under standard conditions.

III.RESULTS AND DISCUSSION

A. Polymorphism in the Parental Lines

Screening and identifying the off-types plays a crucial step in determining the purity of F1 hybrids (Lee et al, 2011). The presence of these impurities could pose a threat for marketing to any seed production companies and also could end up in paying huge compensation to farmers. It is mandatory to maintain the genetic purity of hybrid seed for the successful crop production, as the field GOT is laborious, time consuming and also easily influenced by environmental factors. Alternatively, many DNA markers had been used till date for identifying the off-types in most of the crop species. The uses of molecular markers are always found to be advantages over biochemical or morphological methods to obtain distinct genotype specific profiles. As biochemical markers are easily influenced by external factors, they exhibit limited polymorphism with no discrimination between closely related inbred lines (Lucchese *et al.,* 1999) and morphological markers are known to be laborious, time consuming and environmentally influenced. But, DNA markers can overcome most of these disadvantages and can be very useful to distinguish varieties and off types in very

little time with more accuracy. The importance of Molecular fingerprinting technique and assessment of genetic purity of hybrid seeds using microsatellite markers was established by Nandakumar N, et. al., (2004) in rice.

The present investigation uses SSR marker for identification of off-types in five commercially important vegetable crops. The hybrids of these crops along with their parental lines, revealed that this technique of SSR markers can be successfully used to identify the off-types present in a seed lot. Since SSR is a co-dominant marker and has high polymorphic information content (PIC) value than most of other DNA markers, the application of microsatellites in fingerprinting has been promoted as molecular markers (Ashikawa *et al.*, 1999). So this can be used as powerful tool for screening out off-types and determine the genetic purity.

In the present study, 21 Chilli pepper primers (solgenomics.net), 18 Tomato primers (solgenomics.net), 20 Okra primers (Schafleitner et al., 2013),16 Cucumber primers (cucurbitgenomics.org) and 21 Bitter gourd primers (cucurbitgenomics.org), collectively 96 SSR's primer pairs associated with each specific hybrid and parental lines were assessed. The PCR products of the samples were resolved on 3.5% agarose gel, visibly showed polymorphism between the parental lines (Male and Female) and the corresponding banding pattern was exhibited by the hybrids and any deviation from the banding pattern confirmed the off-types in sample lot.

Among the selected 96 SSR markers studied, one polymorphic primer having high PIC value was identified for each crop, the sequence information of the polymorphic primers are as shown in Table II and rest of primers showed monomorphic banding pattern. The banding pattern of parental lines of Chilli pepper, Tomato, Okra, Cucumber and Bitter gourd, that showed polymorphism in parental lines and its respective hybrids are recorded. The amplified sizes of allele of each primer for male and female parents of all the five crops along with their respective hybrids are summarized in Table II.

TABLE II
Details Of Primer Sequence And Its Amplified Allele Size

CROP	VARIETY NAME		AMPLIFIED BAND SIZE (bp)	PRIMER SEQUENCE (5' to 3')
Chilli pepper	Female	CAF-01	230	F – TGCATTGGTGGGCTAACATA
	Male	CAM-01	250	R - GCTCTTGACACAACCCCAAT
Tomato	Female	LEF-02	230	F - CGATTAGAGAATGTCCCACAG
	Male	LEM-02	250	R - TTACACATACAAATATACATAGTCTG
Okra	Female	AEF-03	250	F - GGCAACTTCGTAATTTCCTA
	Male	AEM-03	220	R - TGAGTAAAAGTGGGGTCTGT
Cucumber	Female	CSF-04	210	F - TCGCCCACGTCCTCTATATC
	Male	CSM-04	190	R - GCTAATGAAGGGGGAGGAGA
Bitter gourd	Female	MCF-05	230	F - CTCCAACTTGAGGAAAGAAAAC
	Male	MCM-05	200	R - AGAGCCAATTGGGGCTTTAT

To assess the off-types percentage, the amplified PCR product of parental line (Male and female) and their respective hybrids samples of each crop were resolved by electrophoresis in 3.5 % agarose gel. The banding pattern of hybrids was assessed corresponding to the parental line and inference was drawn for off-types percentage Table III.

PCR product having no DNA was kept as negative control and 100bp DNA ladder was used as size marker. The banding pattern of hybrids in chilli pepper, (Fig.1) exhibited 13 off-types accounting to 13.27 % of impurities present in the given seed lot. Similarly, Tomato (Fig.2) with 1, okra (Fig.3) with 1, cucumber (Fig.4) with 3 and bitter gourd (Fig.5) with 6 off-types accounting to 1.03 %, 1.02 %, 3.06 %, and 6.12 % impurities respectively. The number of male and female off-types was also recorded based on the presence of single allele in hybrids corresponding to the respective parent. The percentages of off-types were calculated based on the formula as below.

$$\% \text{ off-types} = \frac{\text{Total number off-types}}{\text{Total number of samples tested}} \times 100$$

TABLE III
Percentage Of Hybrid Purity Of The Crops

Crop	Variety name		No. of Female Off-types	No. of Male Off-types	Off-types %	Hybrid Purity %
	Male	Female				
Chilli pepper	CAM-01	CAF-01	1	12	13.27	86.73
Tomato	LEM-02	LEF-02	0	1	1.03	98.97
Okra	AEM-03	AEF-03	0	1	1.02	98.98
Cucumber	CSM-04	CSM-04	2	1	3.06	96.94
Bitter gourd	MCM-05	MCF-05	1	5	6.12	93.88

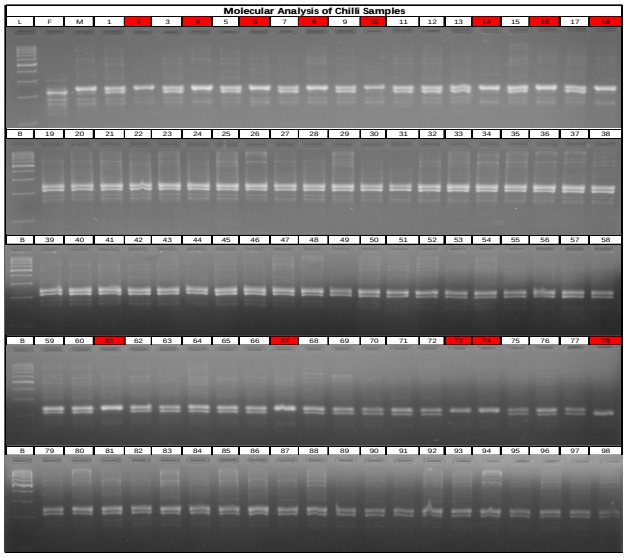

Fig. 1 Molecular Analysis of Chilli samples

Note: Lane L – 100bp DNA Ladder; Lane F – Female parent; Lane M – Male parent; Lane E – Empty; Lane B – Blank (negative control); Lane 1- 98 – F1 individual samples of HyChi – 01 and lanes marked red are off-types

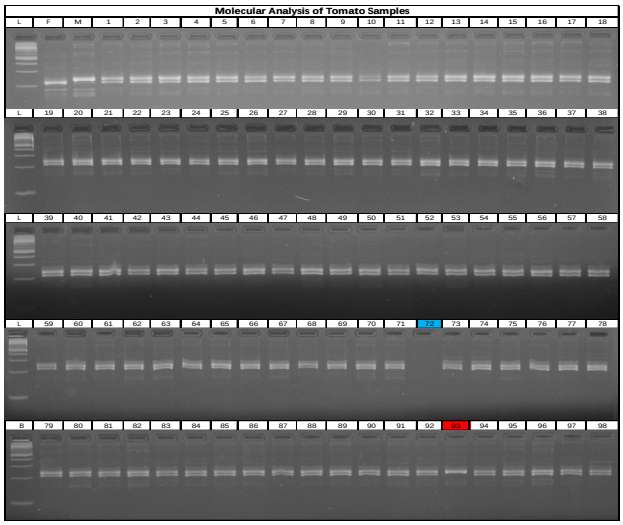

Fig. 2 Molecular Analysis of Tomato samples

Note: Lane L – 100bp DNA Ladder; Lane F – Female parent; Lane M – Male parent; Lane E – Empty; Lane B – Blank (negative control); Lane 1- 98 – F1 individual samples of HyTom – 02, lanes marked red are off-types and blue are non-amplified

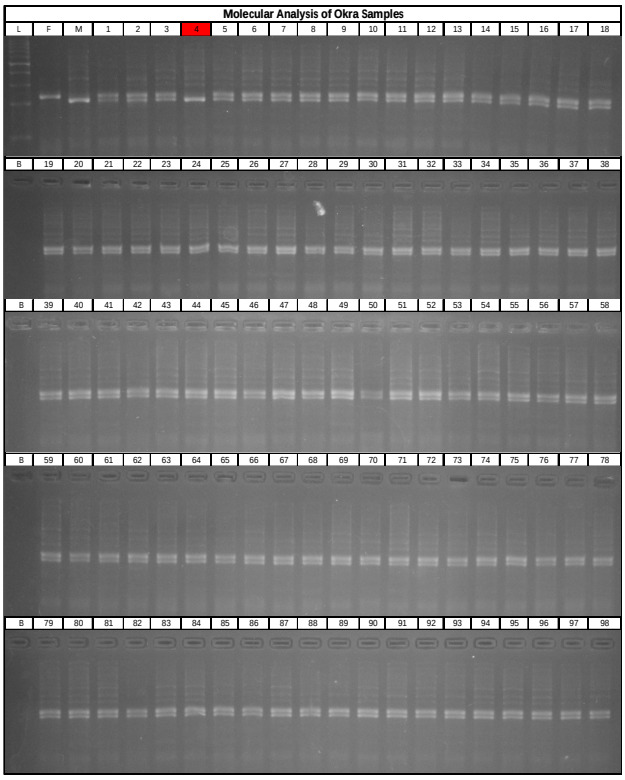

Fig. 3 Molecular Analysis of Okra samples

Note: Lane L – 100bp DNA Ladder; Lane F – Female parent; Lane M – Male parent; Lane E – Empty; Lane B – Blank (negative control); Lane 1- 98 – F1 individual samples of HyOk – 03 and lanes marked red are off-types

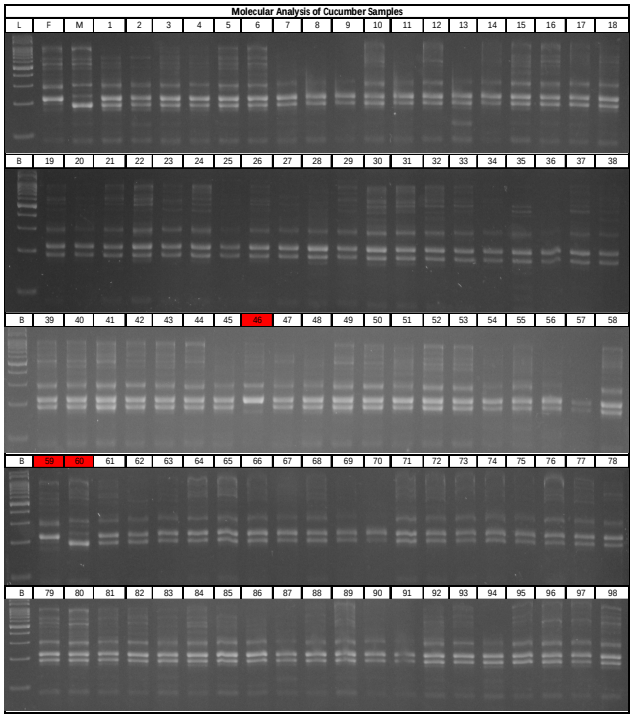

Fig. 4 Molecular Analysis of Cucumber samples

Note: Lane L – 100bp DNA Ladder; Lane F – Female parent; Lane M – Male parent; Lane E – Empty; Lane B – Blank (negative control); Lane 1- 98 – F1 individual samples of HyCum – 04 and lanes marked red are off-types

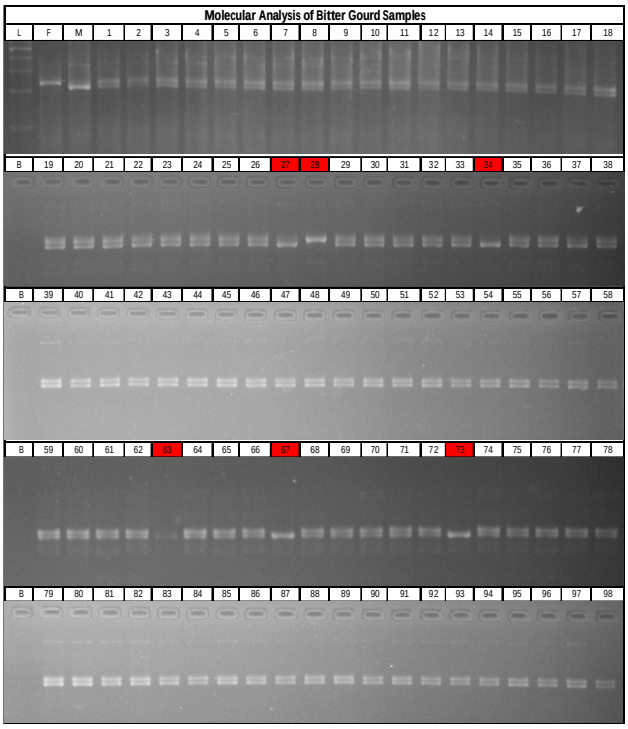

Fig. 5 Molecular Analysis of Bitter Gourd samples

Note: Lane L – 100bp DNA Ladder; Lane F – Female parent; Lane M – Male parent; Lane E – Empty; Lane B – Blank (negative control); Lane 1- 98 – F1 individual samples of HyBit – 05 and lanes marked red are off-types

IV. CONCLUSION

Based on the banding pattern, among 93 samples in each crop variety, at least one off-type was observed. The off-type plants exhibited single allelic band either corresponding to Male or female specific allele, inferring it as an off-types specific to either of the sex. Similarly, majority of the plants amplified two alleles corresponding exactly at the same size of allele as their parents; the presence of both female and male parent alleles in the samples confirms the hybridity (F1 hybrid). The present study clearly demonstrated that SSR's are highly reliable markers for quick and effective in identifying the off-types. Hence, SSR markers can be used for regular screening for off-types and determine the genetic impurity of the crop for a given commercial lot.

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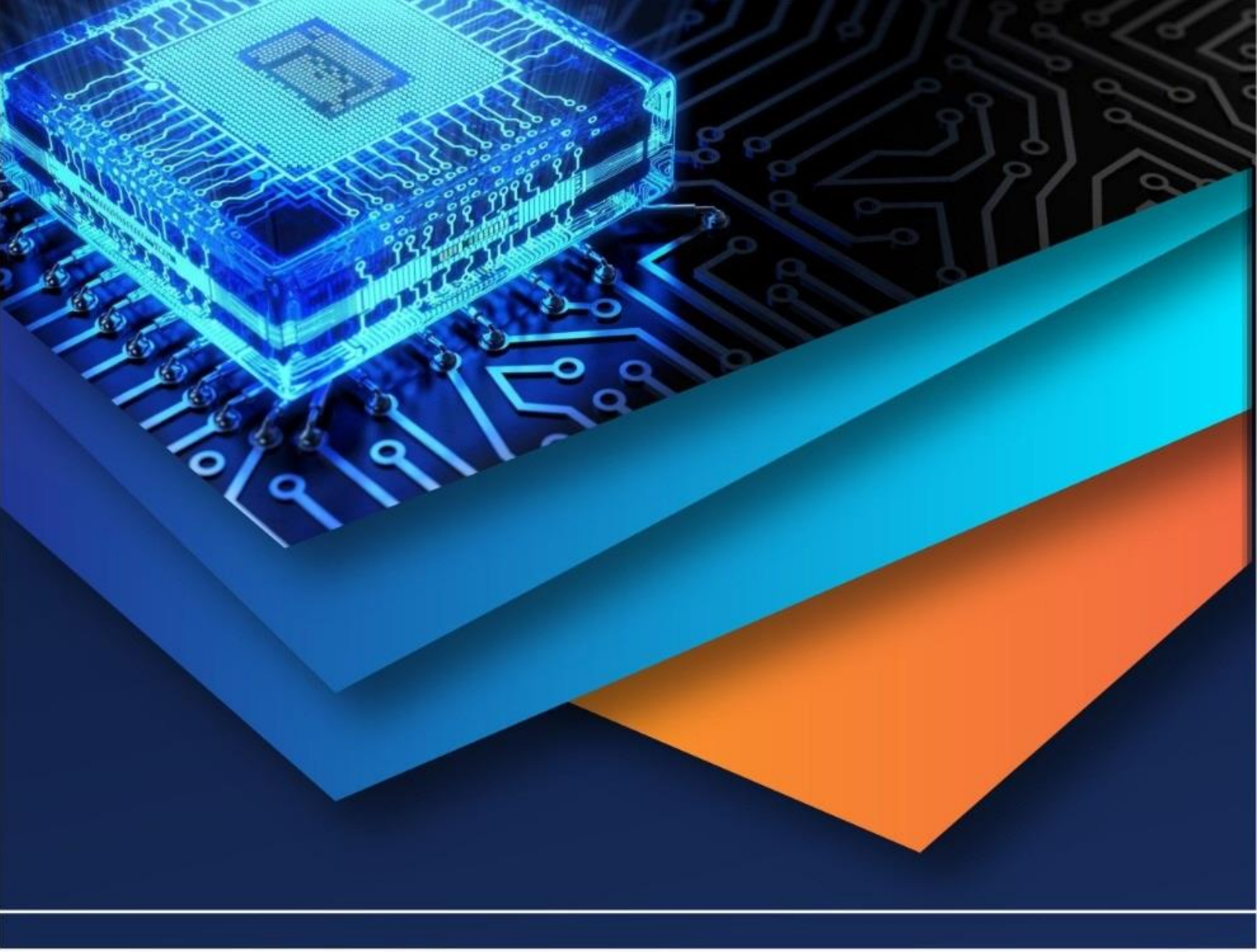

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