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GENETIC VARIATIONS OF *IN VITRO* PROPAGATED MD2 PINEAPPLES USING DNA-BASED MARKERS

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Abstract: Genetic variation determinations among micro-propagated plants is often tested to prevent the occurrence of morphological abnormalities which are deemed unsuitable for commercial use. Although the MD2 pineapple has been widely propagated clonally, there is no genetic fidelity testing on the micro-propagated MD2 pineapple. This study aims to determine genetic variations among the micro-propagated MD2 pineapples using Randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers. The amplification of both markers across 22 pineapple genotypes generated 120 bands with polymorphism percentages of between 93% and 73%, respectively. The evaluation through Rp, PIC and MI showed RAPD more informative (Rp = 7.08, PIC = 0.34, MI = 1.72) compared to ISSR (Rp = 4.17, PIC = 0.32, MI = 1.00). The PCA resulted in indefinite clustering patterns while the dendrograms portray the inability of the markers to correlate the plant morphology with their genetic structure. An Analysis of molecular variance (AMOVA) analysis found high genetic variation (>70%) within the pineapples under review. Meanwhile, the Phi statistic estimate showed a wide genetic variation among the studied genotypes. To conclude, both markers suggest that the plant morphology did not inevitably correlate to its genetic structure. This study may form the basis for the MD2 breeding programme and explore other molecular markers that can potentially correspond to phenotypic polymorphisms.

Keywords: Pineapple, genetic variation, tissue culture, RAPD, ISSR, UPGMA.
Abbreviations: AMOVA: Analysis of molecular variance, FAMD: Fingerprint analysis with missing data, ISSR: Inter-simple sequence repeats, PAST: Paleontological statistics, PCA: Principal component analysis, PCA: Principal component analysis, PIC: Polymorphism information content, RAPD: Random amplified polymorphic DNA, UPGMA: Unweighted pair group with arithmetic average.

Introduction

Plant tissue culture offers a substitute propagation method which enable vast production of plant clones identical to its mother plant and this technique may provide the key answer in battling world hunger (Krishna *et al.*, 2016; Ghosh *et al.*, 2021). Unfortunately, this technique often results in abnormalities/ variation among its regenerants. Evidently, plant propagation processes under laboratory conditions such as tissue wounding and callus dedifferentiation triggers genomic shock resulting in chromosomal changes, methylation, DNA demethylation and the activation of various transposons. The genomic shock triggers the alteration of DNA sequence and induce genetic variation in *in vitro* regenerants (Krishna *et al.*, 2016; Leva & Rinaldi, 2017; Bednarek & Orłowska, 2020; Ghosh *et al.*, 2021; Ranghoo-Sanmukhiya, 2021).

The MD2 pineapple is currently the most demanded pineapple variety in the market thanks to its superior quality. The high demand of this fruit variant resulted in a gradual shift from Smooth Cayenne (SC) to MD2 by pineapple growers making the traditional propagation via slips and suckers insufficient to meet consumer demand.

Tissue culture techniques were proposed as a means to increase disease-free and uniform planting material supply for farmers. Over the years, numerous studies were conducted to find a suitable tissue culture medium (Danso *et al.*, 2008; Teh *et al.*, 2013; Usman *et al.*, 2013; Wan Rizzal *et al.*, 2014; Halim *et al.*, 2018) and shoot proliferation technique (Hamid *et al.*, 2013; Ab Rahman *et al.*, 2019) for mass production of MD2 pineapple plantlets via *in vitro*.

However, abnormalities such as spiny leaves (Sanewski, 2020), double or multiple crowns, conical fruit shapes (Noor Baiti *et al.*, 2017) and dwarf plants (Halim *et al.*, 2018) were often reported in tissue-cultured MD2 pineapples. Thus, owing to the somaclonal variation phenomenon, the condition made farmers averse to tissue culture techniques and many farmers suffered economic loss as it hinders vast production of genetically uniform plants leading many growers to give negative feedback.

Therefore, early detection on somaclonal variations in clonally propagated propagules is crucial to eliminate undesirable variations and to reduce losses for growers. There have been no studies on early somaclonal variation in clonally-raised pineapples. In fact, the detection of the plant abnormalities depends solely on morphological data, which is time-consuming and is highly influenced by environmental factors and the developmental stages of the plant (Krishna *et al.*, 2016; Roostika *et al.*, 2016; Kohpaii *et al.*, 2017, Shaban *et al.*, 2022).

To date, there are numerous stable and efficient molecular markers have been developed. Studies on genetic diversity using molecular marker such as simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism, (RFLP) are applied in pineapple studies (Zhao & Qin, 2018). However, these methods shown some disadvantages such as high cost in AFLP technique and radioactive labelling in RFLP (Arti *et al.*, 2018). In SSR markers, despite being highly polymorphic and ubiquitously distributed in the genome, the design of the marker is heavily relied on prior information of DNA sequence flaking motif (Sønstebø *et al.*, 2007; Jaiswal *et al.*, 2017) and somewhat shown lower congruence with dominant marker data (Liu *et al.*, 2016). Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers are very useful in establishing genetic stability of micro-propagated crops.

ISSR markers are effective in uncovering polymorphism and giving composite marker patterns. In contrast, RAPD markers generated high fragments number (Singh *et al.*, 2014; Ganie *et al.*, 2015).

In the past, molecular analysis recruiting RAPD and ISSR markers are widely used to investigate genetic diversity and phylogenetic relationships among plants. For example, RAPD and ISSR markers were applied on to micro-propagated plants such as *Ananas comosus* (Roostika *et al.*, 2015), *Neolamarckia cadamba* (Pei-Kieng & Wei-Seng, 2019), *Rhododendron mucronulatum* Turcz (Novikova *et al.*, 2020), *Aristolochia indica* (Dey *et al.*, 2021), *Dendrobium fimbiratum* (Tikendra *et al.*, 2021) and *Rhynchostylis retusa* (L.) (Oliya *et al.*, 2021). Apart from that, RAPD and ISSR markers appears to be the first choice for genetic variation studies among plants. Previous assessment of genetic diversity research works on pineapple were conducted on pineapple accessions using ISSR (Vanijajiva, 2012; Souza *et al.*, 2017; Wang *et al.*, 2017; Silva *et al.*, 2019; Harahap *et al.*, 2021), RAPD (Ruas *et al.*, 2001; Santos *et al.*, 2008). In addition, linkage mapping of pineapple was constructed via RAPD, AFLP and ISSR markers (Carlier *et al.*, 2004).

In this study, RAPD and ISSR markers were selected for their particular advantages of being straightforward in their application, relatively cheap and are highly sensitive to genomic polymorphism (Giachino, 2019; Ho *et al.*, 2021; Murthy *et al.*, 2021).

This paper makes a comparison and selection of the most informative marker system between RAPD and ISSR in evaluating genetic variation in MD2 pineapple plants and assessing

genetic diversity among clonally propagated-field cultivated MD2 pineapple genotypes.

Materials and Methods

Plant Materials

A total of 21 MD2 plant regenerants and one conventionally cultivated SC were collected from the CRAUN Research Sdn Bhd research site located at Kampung Mang in Kota Samarahan, Sarawak, Malaysia. The list of the plant with its morphology is presented in Table 1. The leaf samples were washed with tap water to remove dirt and soil followed by rinsing with distilled water before being kept in individual zip-lock bags and stored at -20°C for future use.

Table 1: List of MD2 pineapple plants and its morphological features

Figure Code	Sample Code	Lane Number on Gel	Morphology
A	W1 W2 W3 NTC1 NTC2 NTC3	2 3 4 5 6 7	Normal fruit size Normal crown size Normal leaves
B	AB1 (1) AB1 (2) AB1 (3)	8 9 10	Normal fruit size Small crown size Thorny leaves
C	AB2 (1) AB2 (2)	11 12	Normal fruit size Small crown size Normal leaves
D	AB3 (1)	13	Non-fruiting Normal leaves
E	AB3 (2) AB3 (3)	14 15	Non-fruiting Thorny leaves
E	AB4 (1)*	16	Non-fruiting Thorny leaves
E	AB4 (2)*	17	Non-fruiting Normal leaves
E	AB4 (3)*	18	Non-fruiting Thorny leaves
F	AB5 (1) AB5 (2)	19 20	Small fruit size Small crown size Normal leaves
G	AB6 (1)	21	Small fruit size Normal crown size Normal leaves
H	AB6 (2)	22	Small fruit size Normal crown size Normal leaves Produce slips

W: Normal traditional cultivated plant, NTC: Normal tissue cultured plant, Ab: Abnormal plants. Note that normal MD2 pineapple leaves have thorns at its margin whereby thorny leaves have thorns from the base till the tip of the leaf. Normal crown size: 50-150% from the length from the length of the fruit. Plant samples with * were collected at the flooded area

DNA Isolation and Quantification

DNA was extracted following the extraction technique described by Dellaporta *et al.* (1983). An approximate 1 g of basal (white part) of the pineapple leaf was ground into fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred into a clean 50 mL round bottom centrifuge tube and mixed with 15 mL pre-warmed (65°C) extraction buffer [0.10 M Tris-HCl (pH 8.0), 0.50 M NaCl, 50 mM EDTA (pH 8.0), 2% v/v ß-mercaptoethanol] (Dellaporta *et al.*,1983).

The mixture was incubated at 65°C water bath for 30 minutes with intermittent mixing. A volume of 15 mL chloroform-isoamyl alcohol (24:1) was added and the mixture was subjected to centrifugation at 16,100 x g for 15 minutes in a refrigerated centrifuge at 4°C. The precipitation of nucleic acid was done using ice-cold isopropanol (1:1v/v) overnight at - 20°C and the DNA pellets were collected by centrifugation at 16,100 x g for 15 minutes at 4°C.

The DNA pellets were washed twice with 1 mL 70% cold ethanol, air-dried and resuspended in a 100 µL TE buffer [10 mM Tris HCl pH 8.0 and 1 mM EDTA (pH 8.0)]. An amount of 0.2 µL of RNase was added for RNA removal treatment. The DNA sample was stored at -20°C for future use. The purity and the concentration of DNA samples were quantified using UV spectrophotometer (Eppendorf BioPhotometer D30, Germany). The crude DNA extracts were diluted to make a working solution of 100 ng/ µl.

PCR Amplification

A total of 10 RAPD and 8 ISSR primers purchased from the Integrated DNA Technologies (IDT) were screened for their clear banding and repeatable amplifications. For each reaction, 15µl amplification mixtures containing 100 ng of template DNA, 10 mM of each dNTPs (dATP, dTTP, dCTP and dGTP in 1:1:1:1 parts), 0.24 µl of Taq DNA polymerase, 2 µl of each primer and 25 mM of $MgCl_2$ were prepared.

The amplification was performed according to the PCR programme parameters: Initial denaturation at 94°C for 2 minutes followed by 45 cycles of denaturation at 94°C for 45 seconds, annealing for 30 seconds, extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The annealing temperature of each marker was re-adjusted based on the GC content of the primer obtained from the OligoAnalyzer® program at www.idtna.com/scitools. The amplified PCR products were resolved on 12% polyacrylamide gel electrophoresis (PAGE) for 2 hours at 85 V using 1 Kb DNA ladder (Thermo Scientific, Germany) as marker. The gel photographs were taken using a gel documentation system (MultiDoc-It™ System, UVP).

Marker Scoring and Primer Informativeness Analysis

The RAPD and ISSR band profiles were conservatively scored with the aid of Python-based software, PyElph with binary character representing (1) for present band and (0) for absent band. The software assists in band matching and detection through band migration that matches from all plant samples (Pavel & Vasile, 2012).

Primer Informativeness were evaluated through different parameters such as PIC, E, MI and Rp to measure the polymorphism information and discriminatory power for individual markers to resolve genetic variations among MD2 genotypes. The parameters (PIC, E, MI and Rp) were calculated using a user-friendly interface program known as Online Marker Efficiency Calculator (iMEC) available at https://irscope.shinyapps.io/iMEC/.

Statistical Analysis

The data matrix obtained from both markers were converted into genetic similarity index using Jaccard similarity coefficient in PAST (Paleontological Statistic version 3.24) (Hammer *et al.*, 2001) software package. The resultant similarity indexes were used to performed principal component analysis (PCA), Unweighted Pair Group Method with Arithmetic Average (UPGMA) and analysis of molecular variance (AMOVA).

The genetic relatedness of among the 22 genotypes experimented were analysed using PCA through Microsoft Excel add-in, XLSTAT version 2019.1 and UPGMA with 1,000 bootstrap replications using a PAST software package. The detail on genetic constituent between and within MD2 plants was evaluated using AMOVA via the Fingerprint Analysis with Missing Data version 1.3 (FAMD) software (Schlüter & Harris, 2006).

Results and Discussion

RAPD and ISSR Binding Profiles

A total of six RAPD markers (OPA-03, OPA-07, OPA-11, OPA-13, OPA-18, OPA-19) and six ISSR markers (ISSR-1, ISSR-3, ISSR 4, ISSR-6, ISSR-7, ISSR-8) were selected from 18 markers based on their repeatability and clear banding pattern to analyse the entire set of 22 phenotypes in the study (Figure 1).

The bands fragments were conservatively scored where weak or ambiguous bands were excluded to increase the fragments' confidence level (Costa *et al.*, 2016). Upon amplification, a total of 213 loci were generated by both RAPD and ISSR primer sets.

The PCR amplifications using RAPD marker system generated a total of 120 DNA fragments across 22 genotypes studied with number of amplified fragments varying from 11 bands (OPA-18) to 30 bands (OPA-03) with band size ranging between 100 and 2,500 bp. Out of the 120 amplified fragments, 112 (93.33%) bands were polymorphic, with an average value of 18.67 bands per primer. The highest PIC value in RAPD primers was denoted by OPA-11 (0.37) and the lowest represents by OPA-03 (0.29) with the average PIC value at 0.338. The Rp values for the markers were found to be between 2.00 and 12.18 for OPA-18 and OPA-19 respectively, across all genotypes (Table 2).

The ISSR marker system PCR amplification produced a total of 93 scorable band fragments of which 68 were polymorphic in nature accounting for 73.12% polymorphism with average value at 11.33 bands per primer. The number of amplified fragments ranged from 9 bands (ISSR-6) to 22 bands (ISSR-8) which varied in size between 80 and 2,500 bp. The PIC value varied from 0.27

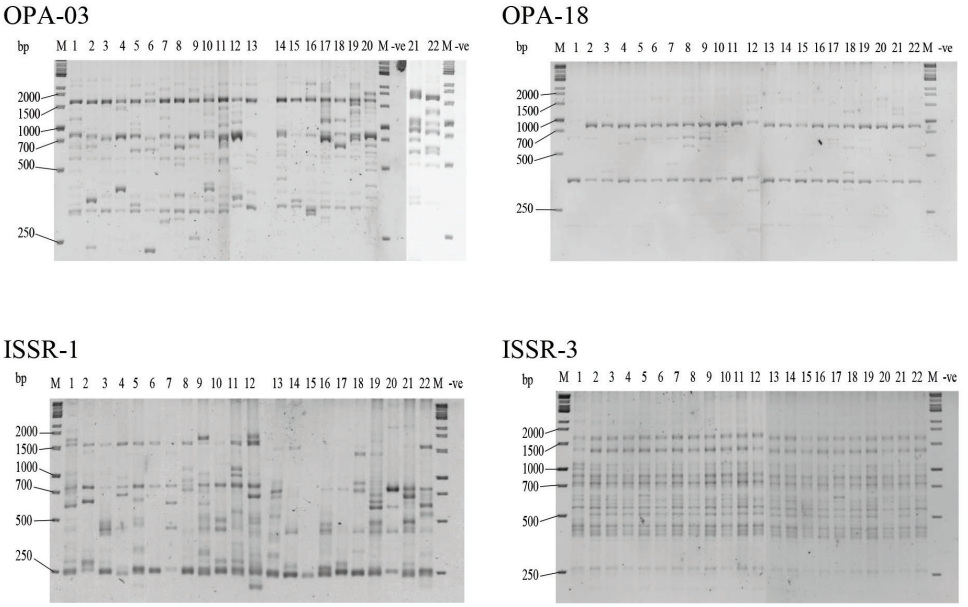


Figure 1: The band profile of 22 genotypes generated from RAPD and ISSR markers. Lane M represents the 1 kb DNA ladder. Lane 1 represents SC. Lanes 2-22 represents different MD2 pineapple phenotypes as listed in Table 1 while -ve represents the negative control

(ISSR-6) to 0.38 (ISSR-8) with an average value at 0.32. The ISSR set for Rp value recorded the highest at 8.36 for ISSR-1 and the lowest at 1.27 for ISSR-6 (Table 2).

Theoretically, the tenacity of a marker to resolve genetic variation between genotypes is directly related to the total and the average of polymorphic percentage as well as the average polymorphic bands per primer (PB/B) (Giachino, 2019; Oliveira *et al.*, 2019). In this study, the RAPD markers displayed high polymorphism levels of up to 93.33% with average 18.67 polymorphic bands per primer. Meanwhile, the ISSR molecular markers detected polymorphism at 73.10% across 22 genotypes with average polymorphism of 11.33 polymorphic bands per primer.

Similar results were reported in the studies involving *Robinia ambigua 'idahoensis'* (Ngezahayo *et al.*, 2007), *Dalbergia sissoo* (Arif *et al.*, 2009), yarrow (Farajpour *et al.*, 2011), south Tunisian barley (Guasmi *et al.*, 2012), *Codonopsis lanceolate* Benth. et Hook. F. (Guo *et al.*, 2014), *Saccharina* (Cui *et al.*, 2016) and *Nilgirianthus ciliates* (Rameshkumar *et al.*, 2019) which reported that RAPD markers produced more polymorphic bands compared to ISSR molecular marker.

Comparatively, the data reported in PQM-1 pineapple (Prakash *et al.*, 2009), *Cicer arietinum* and *Cajanus cajan* L. (Lal *et al.*, 2010), *Bacopa monnieri* (Muthiah *et al.*, 2013), *Cymbopogon* (Baruah *et al.*, 2016), *Croton tetradenius* Baill (Almeida-Pereira *et al.*, 2017) and *Dendrobium chyrysotoxum* Lindl (Tikendra *et al.*, 2019) reported that the ISSR test resulted in higher polymorphism percentages.

Table 2: Comparison of RAPD and ISSR markers informativeness in studying genetic variation among different pineapple genotypes

Primer	TNB[a]	NMB[b]	NPB[c]	% Polymorphism	Band Size (bp)	PIC[d]	Rp[e]
RAPD							
OPA-03	30	1	29	96.667	250-2500	0.289	10.636
OPA-07	24	2	22	91.667	250-3000	0.353	9.273
OPA-11	13	3	10	76.923	100-1000	0.372	2.455
OPA-13	16	1	15	93.750	100-1000	0.349	5.909
OPA-18	11	1	10	90.909	300-1250	0.313	2.000
OPA-19	26	0	26	100.000	250-2000	0.349	12.182
Total	120	8	112	-	-	2.026	-
Average	20	1.333	18.667	93.333	158-1791	0.338	7.076
ISSR							
ISSR-1	21	1	20	95.238	200-1500	0.303	8.364
ISSR-3	14	7	7	50.000	250-2000	0.275	4.000
ISSR-4	12	4	8	66.667	300-1000	0.374	2.818
ISSR-6	9	4	5	55.556	250-1000	0.270	1.273
ISSR-7	15	4	11	73.333	250-2000	0.335	2.455
ISSR-8	22	5	17	77.273	80-2500	0.375	6.091
Total	93	25	68	-	-	1.932	-
Average	15.5	4.167	11.333	73.118	158-1791	0.322	4.167

[a] Total number of bands, [b]monomorphic bands, [c]polymorphic bands, [d]polymorphism information content, [e]resolution power

As for the average polymorphism per primer, the RAPD test produced higher average polymorphism per primer results at 18.67 compared with ISSR markers at only 11.33 counts of polymorphism per primer. Reports with similar results where RAPD provided higher average polymorphic bands per primer compared ISSR markers were reported in the genetic diversity studies involving yarrow (10.77 PB/P vs 10.14 PB/P) (Farajpour *et al.*, 2011), South Tunisian barley (5.66 PB/P vs 3 PB/P) (Guasmi *et al.*, 2012) and *Crocus sativus* L. (10.73 PB/P vs 9.50 PB/P) (Mir *et al.*, 2021).

The extremely high average polymorphic bands per primer generated by RAPD markers in this study were also reported in *Citrullus lanatus* (12.2 PB/P) (Mujaju *et al.*, 2010), *Hordeum vulgare* (13.87 PB/P) (Eshghi *et al.*, 2012) and banana (32.86 PB/P) (Singh *et al.*, 2021).

However, a study on *Vigna radiata* (Singh *et al.*, 2012) and sugar beet (Izzatullayeva, 2014) reported that ISSR markers gave extremely high average polymorphic bands per primer of 16.87 PB/P and 14.4 PB/B, respectively compared with their counterpart markers.

Another essential feature of an excellent marker system is the Resolving power (Rp). The Rp is described as the overall capacity of a molecular marker to distinguish samples and is commonly correlates to the genotype discrimination (Prevost & Wilkinson, 1999; Guasmi *et al.*, 2012; Verma *et al.*, 2017). In this study, the average Rp value demonstrated by RAPD was 7.08 compared to ISSR average Rp value at 4.17. Previous studies on South Tunisian barley (Guasmi *et al.*, 2012) and ginger (Baruah *et al.*, 2019) reported similar results where the RAPD Rp value was higher than ISSR value (RAPD Rp = 2.99, ISSR Rp = 0.92) and (RAPD Rp = 4.41, ISSR Rp = 3.20), respectively.

The RAPD molecular markers resulted in higher reading in their PIC, E and MI (PIC = 0.34, E = 5.09 and MI = 1.72) compared to ISSR molecular markers with (PIC = 0.32, E = 3.09 and MI = 1.00). This finding was comparable to genetic characterization of *Cannabis sativa* L. (RAPD PIC = 0.28, ISSR PIC = 0.25) (Kayis *et al.*, 2010), micro-propagated pistachio (RAPD PIC = 0.23, ISSR PIC = 0.22) (Akdemir *et al.*, 2016) and *Pimpinella anisum* L. (RAPD PIC = 0.28, ISSR PIC = 0.25) (Giachino, 2019). Similarly, higher MI readings were denoted in RAPD marker systems compared with ISSR markers with MI value of 3.69 and 2.60, respectively in the study of ginger germplasm (Baruah *et al.*, 2019).

In this study, the RAPD molecular markers generated a higher average total number of polymorphic bands, average polymorphism percentage and average polymorphic bands per primer compared with the ISSR molecular marker system across 22 genotypes despite ISSR molecular markers are profoundly reported to be more effective in detecting polymorphism compared to RAPD marker system (Singh *et al.*, 2012; Verma *et al.*, 2017).

ISSR markers are better than RAPD at detecting polymorphism because the former has a better capacity to generate several informative bands within a single amplification and exhibited better classification in intra- and inter-genomic diversity other than the arbitrary primers (Karuppanapandian *et al.*, 2010).

Some reports, however, noted that RAPD markers were more effective in detecting polymorphism than the ISSR marker systems (Ebrahimi *et al.*, 2012; Guasmi *et al.*, 2012; Guo *et al.*, 2014; Patel *et al.*, 2016; Rameshkumar *et al.*, 2019). The high total polymorphic bands, polymorphism percentage and the average polymorphic band fragments per primer generated by RAPD markers were the result of the marker's location in the coding and non-coding sequences within the genome of the experimented species.

The residing of the marker at the non-coding region consequently increases the polymorphic band number detection and the polymorphic band fragments per primer (Loarce *et al.*, 1996; Saleh, 2011; Costa *et al.*, 2016; Verma *et al.*, 2017). In comparison, the high polymorphism percentage reported in ISSR markers reported in pineapple (Prakash *et al.*, 2009), *Cicer arietinum* and *Cajanus cajan* L. (Lal *et al.*, 2010), *Bacopa monnieri* (Muthiah *et al.*, 2013), *Cymbopogon*

(Baruah *et al.*, 2016), *Croton tetradenius* Baill (Almeida-Pereira *et al.*, 2017) and *Dendrobium chyrysotoxum* Lindl (Tikendra *et al.*, 2019) were due to the amplification at microsatellite-rich regions of DNA caused by mutations during cell replication (Ghimire *et al.*, 2019).

On the other hand, the low polymorphism percentage demonstrated by the ISSR molecular markers were the result of anchoring the bases at their 3' end subsequently lower the number of sequences in homology to the ISSR markers in producing distinct and specific band fragment (Parson *et al.*, 1997; Saleh, 2011). In general, the difference in polymorphic band numbers detected by the markers is caused by the proportion of the coding and non-coding sequences within the studied species genome composition and the marker's targeting technique (Bublyk *et al.*, 2013; Guo *et al.*, 2014; Wang *et al.*, 2017; Giachino, 2019; Tilwari & Sharma, 2021).

To measure the efficiency of marker system, polymorphic information content (PIC), marker index (MI) and resolving power (Rp) were calculated. The PIC analysis is compulsory in order to identify the most appropriate marker selection for genetic mapping and phylogenetic analysis (Powell *et al.*, 1996).

The PIC values are commonly used as the polymorphism measure for a marker locus using linkage analysis (Patel *et al.*, 2016). Since both RAPD and ISSR marker systems are dominant markers, their maximum PIC value is 0.5 because two alleles per locus are assumed (Bostein *et al.*, 1980; Chesnokov & Artemyeva, 2015; Amiryousefi *et al.*, 2018).

Thus, in referring to the PIC values generated by both RAPD (0.34) and ISSR (0.32) markers indicate appropriate/good polymorphism detection of both markers and have equal potential in differentiating closely related plant samples (Parsons *et al.*, 1997; Nkongolo *et al.*, 2005; Shaw *et al.*, 2009; Lal *et al.*, 2010; Baruah *et al.*, 2016; Tilwari & Sharma, 2021).

Meanwhile, the informativeness of the RAPD and ISSR markers for assessing genetic relationships was evaluated through the MI and Rp (Verma *et al.*, 2017). The MI and E are two analysis used to measure the marker set's ability to provide high polymorphic information content and the general measure to determine the marker systems efficiency in detecting genetic variation of which the MI is the product of PIC and E (Powell *et al.*, 1996; Nagaraju *et al.*, 2001; Grativol *et al.*, 2010). Subsequently, primer which generated higher PIC value will result in higher MI value (Grativol *et al.*, 2010).

As for the Rp value, the high Rp index is correlated to the high polymorphic band number scored (Grativol *et al.*, 2010; Ho *et al.*, 2021). In general, markers with higher polymorphism values will produce higher PIC, MI and Rp values (Verma *et al.*, 2017). Since the PIC, MI and Rp values for RAPD is higher than ISSR, it is suggested RAPD markers are more informative compared with ISSR molecular markers at evaluating genetic fidelity among MD2 pineapple clones.

Genetic Diversity in Ananas comosus var MD2

Jaccard genetic similarity coefficient were generated to observe genetic variant among 22 pineapple genotypes. From the similarity generated by the test, RAPD molecular markers revealed an abnormal type 6 plant individual 1 [AB6 (1)] while the traditionally cultivated plant individual 3 (W3) gave the lowest genetic similarity coefficient value at 0.279. In contrast, the traditional cultivated plant individual 2 (W2) versus traditional cultivated plant individual 1 (W1) showed the highest genetic similarity coefficient value at 0.604.

Using the ISSR marker system, the Jaccard similarity coefficient analysis indicated that abnormal plant type 5 individual 1 [AB5 (1)] and SC scored the lowest similarity value with 0.463. In comparison, the normal tissue-cultured plant 2 (NTC2) scored the highest similarity value with traditional cultivated plant individual 2 (W2) scoring 0.875 similarity value. The pooled data from both RAPD and ISSR primers demonstrated that SC has a distant relationship with the plant sample abnormal type 5 individual 1 [AB5 (1)] with 0.384 similarity coefficient value.

Meanwhile, normal tissue-cultured plant individual 2 (NTC2) and traditional cultivated plant individual 2 (W2) were closely related as they scored the highest similarity value with 0.713. The Jaccard tables revealed a wide genetic variation between pineapple genotypes (RAPD: 0.28 to 0.60, ISSR: 0.46 to 0.88, RAPD & ISSR: 0.42 to 0.71). Similar to the similarity indices value obtained using RAPD and ISSR in the study of *Trigonella foenum-graecum* and *Trigonella caerula* (Dangi *et al.*, 2004), *Euryale ferox* (Kumar *et al.*, 2016), *Zingiber officinale* species (Baruah *et al.*, 2019) and torch ginger (Ismail *et al.*, 2019) reported high range of similarity value among the studied landraces indicating high genetic differences detected among genotypes.

The significance of the difference in the Jaccard similarity values between RAPD (0.28 to 0.60) and ISSR (0.46 to 0.88) supports the different value of polymorphism levels (RAPD = 93.3%, ISSR = 73.3%) detected by both marker systems in the given plant genotypes (Dangi *et al.*, 2004). Furthermore, the difference in genetic levels is attributed to natural selection process due to the accumulation of new gene combinations (Chauhan *et al.*, 2022).

The Jaccard similarity index data represented by the ISSR marker system and the combination markers that were found were able to distinguish between the different pineapple cultivar experimented on in this study where the marker systems revealed high genetic dissimilarity values between MD2 and SC varieties. The ability of ISSR marker system to differentiate different cultivar agrees with previous studies on black gram in which the ISSR marker system technique successfully identified the black gram varieties from the chickpea varieties (Souframanien & Gopalakrishn, 2004; Karuppanadian *et al.*, 2010) and discrimination among seven mung beans (*Vigna*) species (Ajibade *et al.*, 2000; Tabasum *et al.*, 2020).

The ability of ISSR markers to discriminate between different cultivar is greatly influenced by the amplification of repeat sequences at the centromeric region of the plant chromosome whereby the region is predominantly consisting of conserved satellite repeats (Gindullis *et al.*, 2001; Bhardwaj *et al.*, 2010).

By comparison, the RAPD marker system was unable to reveal different pineapple variants but instead detected high genetic dissimilarity between the abnormal type 6 plant individual 1 AB6(1) and traditional cultivated plant individual 3 (W3). The reason for the observed result is because RAPD markers do not sequence conserved satellite repeat at the centromeric region hence RAPD results in diverge genetic similarity between closely related genotypes (Niemann *et al.*, 1997). Moreover, the high polymorphic band number detected by RAPD markers lead to different classifications of plant genotypes (Souframanien & Gopalakrishna, 2004).

For example, in the separation of Kungs II from its close relative Kustro and Halo of rye cultivar was resulted from high polymorphic band number detected by RAPD marker due to the amplification at non-coding region (Loarce *et al.*, 1996). Consequently, a relatively high polymorphic band number produced by RAPD markers would result in the separation of two closely related plant genotypes. Thus, it is highly recommended that the RAPD marker analysis be paired with a more advanced marker system such as ISSR to acquire a more comprehensive and reliable genetic analysis (Bhardwaj *et al.*, 2010; Ghimire *et al.*, 2019; Tikendra *et al.*, 2019).

Additionally, the technique of pooling data from different marker types provides more informative classification compared to the single marker technique because different marker system results in more loci detected due to the amplification at different region of genome (Palombi and Damiano, 2000; Martins *et al.*, 2004; Souframanien & Gopalakrishna, 2004; Karuppanapandian *et al.*, 2010). The exclusion of genetic information by marker system could be minimised with the utilisation of different marker techniques (Ghimire *et al.*, 2019). Therefore, the practice of combining different marker system results in a better analysis of genetic stability/fidelity studies.

Principal Component Analysis (PCA) and Cluster Analysis

With the RAPD system, a PCA analysis revealed seven principal coordinates accounted of more than 60% of the variation, whereas with the ISSR, the PCA analysis explained genetic variation of more than 70%. Meanwhile, the pooled data from both markers showed a total genetic variance of 60% (Table 3).

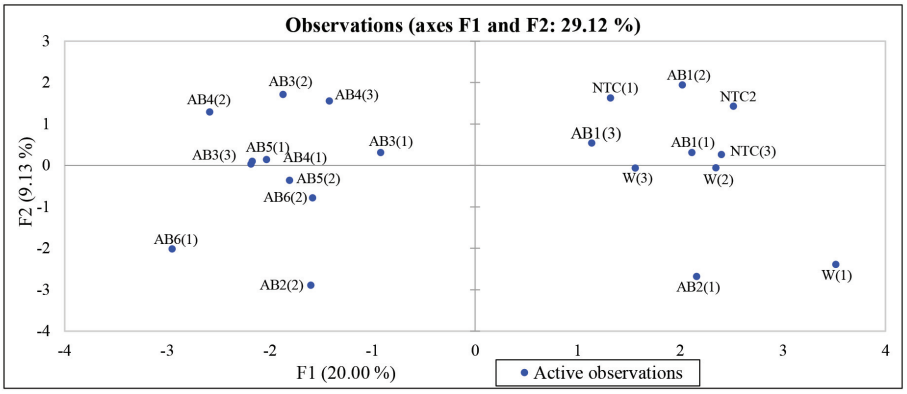
Similarly, the PCA analysis generated by ISSR molecular markers revealed a higher total genetic variation compared with its counterpart marker used in the studies of genetic diversity of black gram (RAPD = 41.81%, ISSR = 43.01%) at the first two principal axes (Karuppanapandian *et al.*, 2010), *Pseudomonas strains* (RAPD = 49.51%, ISSR = 54.62%, RAPD + ISSR = 48.46%) at the first five informative PC components (Rayar *et al.*, 2015) and pineapple plant (SSR = 23.91%, ISSR = 32.3%, SSR + ISSR = 23.21%) at the first three principal components (Wang *et al.*, 2017).

To observe the grouping of pineapple genotypes, PCA plot was generated (Figure 2). The PCA analysis (Figure 2) of the markers were shown unable to group the pineapple genotypes into the same group according to its phenotypes. Thus, to confirm the clustering patterns on the relationship among the plant's genotypes, UPGMA dendrogram was constructed (Figure 3).

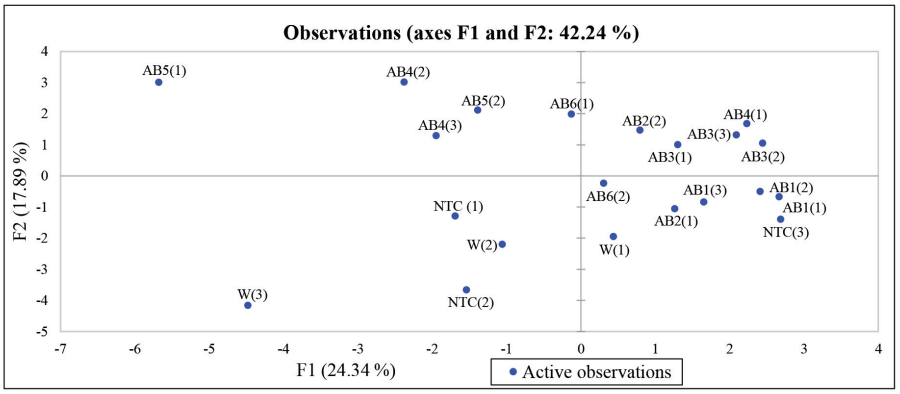
Table 3: PCA informative total variation of each primer and combination primers

Markers	Principal Coordinate	Eigen Value	Variability (%)	Cumulative Variance (%)
RAPD	1.	4.400	19.998	19.998
	2.	2.008	9.125	29.123
	3.	1.791	8.142	37.265
	4.	1.725	7.842	45.107
	5.	1.462	6.644	51.751
	6.	1.332	6.056	57.807
	7.	1.016	4.619	62.427
ISSR	1.	5.356	24.344	24.344
	2.	3.937	17.895	42.239
	3.	2.072	9.419	51.658
	4.	1.444	6.565	58.223
	5.	1.180	5.366	63.589
	6.	1.119	5.085	68.674
	7.	0.895	4.070	72.744
RAPD + ISSR	1.	4.496	20.439	20.439
	2.	2.881	13.095	33.533
	3.	1.614	7.337	40.870
	4.	1.390	6.319	47.189
	5.	1.274	5.791	52.980
	6.	1.155	5.249	58.229
	7.	1.032	4.692	62.921

RAPD



ISSR



RAPD + ISSR

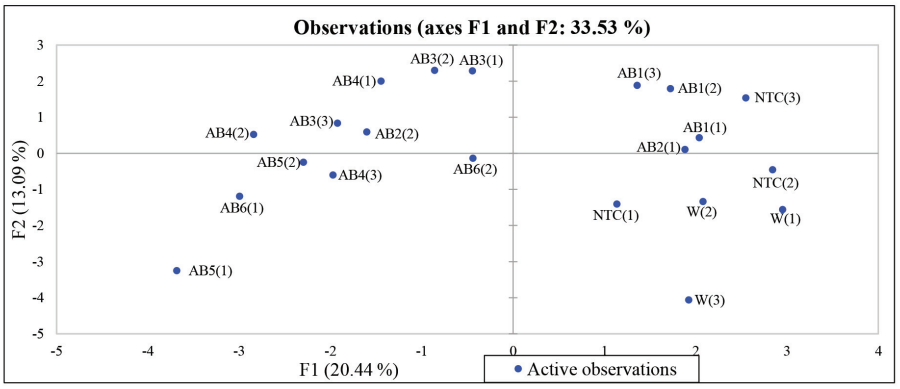


Figure 2: PCA plot of all experimented molecular markers across pineapple genotypes. The first component (X-axis), second component (Y-axis). Clustering patterns generated by RAPD (a), ISSR (b) and combination markers (c). Designations: W, traditional cultivated, NTC, normal pineapple, AB: abnormal sample. RAPD (a), ISSR (b) and combination markers (c)

RAPD-construct phylogenetic tree, all 22 experimented plant genotypes were clustered into three main clusters with the cophenetic correlation coefficient, r = 0.73. The three main clusters were weakly supported by bootstrap value (45).

The dendrogram revealed Cluster I grouped two plant individuals: Abnormal type 5 plant

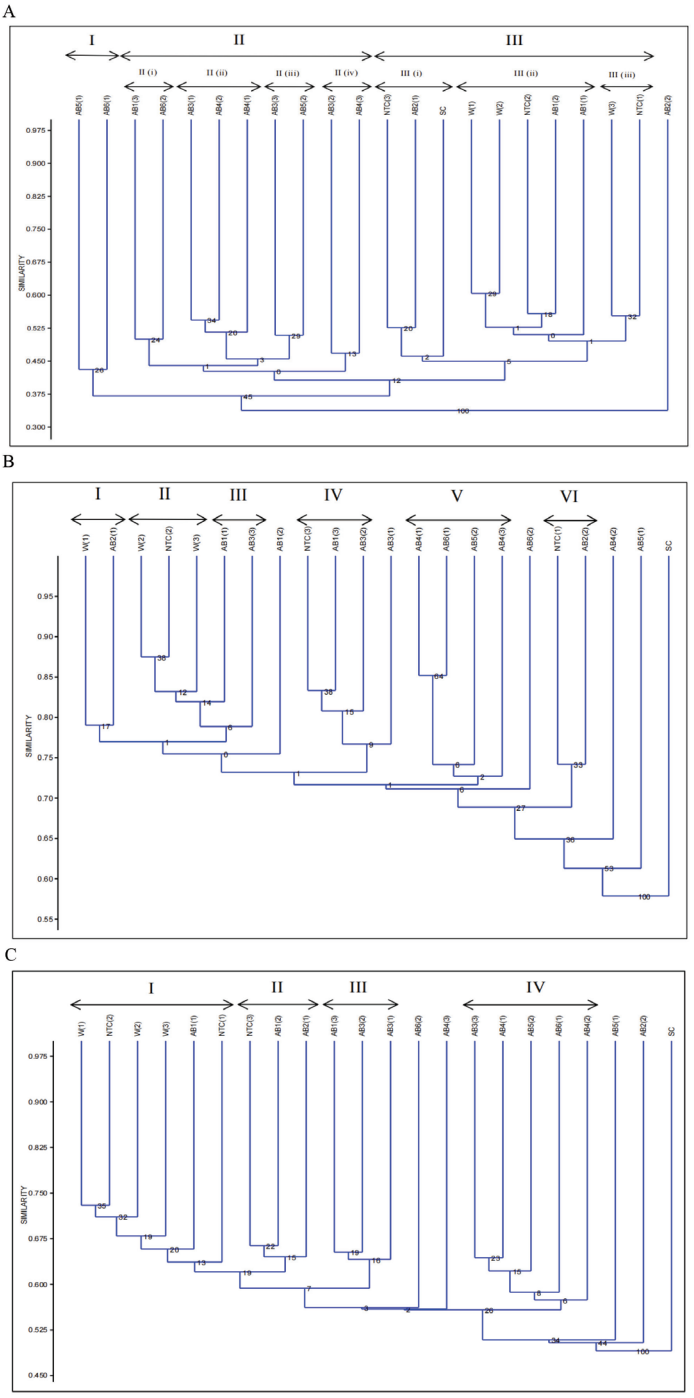


Figure 3: UPGMA dendrogram generated from Jaccard genetic similarity of markers. Phylogenetic analysis across 22 pineapple genotypes based on marker-based genetic similarity RAPD (a), ISSR (b) and combination markers (c). The analysis was performed using PAST software with 1,000 bootstrap replications. Numbers on the node refers to the cluster stability support. W: traditional cultivated, NTC: normal pineapple, AB: abnormal sample, SC: smooth cayenne

individual 1 [AB5 (1)] and abnormal type 6 plant individual 1 [AB6 (1)] at similarity coefficient of 0.520 and at a low bootstrap value of 26. Based on the shared morphological characteristic, the plants portray similarities on small fruit size and normal leaves.

Meanwhile, the Cluster II of the dendrogram comprised nine plant samples which were further divided into four sub-clusters. Plant accession of abnormal type 1 plant individual 3 [AB1 (3)] and abnormal type 6 plant individual 2 [AB6 (2)] were grouped into the first sub-cluster with a support value of 24 with similarity index denoted at 0.500. However, these plant individuals do not possess similar a morphology.

The second sub-cluster which comprised of three plant individuals, abnormal type 3 plant individual 1 [AB3 (1)], abnormal type 4 plant individual 2 [AB4 (2)] and abnormal type 4 individual 1 [AB4 (1)] shared non-fruiting characteristic were tied up at a bootstrap value of 26. The abnormal type 3 plant individual 1 [AB3 (1)] and abnormal type 4 plant individual 2 [AB4 (2)] which were more closely affiliated in the second sub-cluster were related at Jaccard similarity coefficient of 0.537 and they possess non-fruiting and normal leaves phenotypes.

These genotypes were supported at a slightly higher yet weak support value of 34. The abnormal type 3 plant individual 3 [AB3 (3)] and abnormal type 5 plant individual 2 [AB5 (2)] were clustered in the third sub-cluster with Jaccard similarity coefficient at 0.509 and supported at bootstrap value of 29. Similar with AB1 (3) and AB6 (2) plant individuals, the AB3 (3) and AB5 (2) plants do not share similar morphological characteristics.

Lastly, supported at bootstrap value of 13, abnormal type 3 plant individual 2 [AB3 (2)] and abnormal type 4 plant individual 3 [AB4 (3)] were grouped into the fourth sub-clusters with similarity index of 0.521. Referring to their morphology, these plant individuals possess similar characteristics of non-fruiting and having spiny leaves.

In the third cluster, all ten plant samples were further sub-divided into three sub-clusters. The normal tissue-cultured plant individual 3 (NTC3), abnormal type 2 plant individual 1 [AB2 (1)] and SC were grouped into the first sub-cluster with SC having a very weak support (bootstrap value of 2) compared to the bootstrap value (20) at the fork between NTC3 and AB2 (1) plants.

The latter plant affiliates were related at a very low similarity index of 0.283 although possesses similar phenotypic characteristics of normal fruit sizes and normal leaves. Traditional cultivated plant individual 1 (W1), traditional cultivated plant individual 2 (W2), normal tissue cultured plant individual 2 (NTC2), abnormal type 1 plant individual 2 [AB1 (2)] and abnormal type 1 individual 1 [AB1(1)] were clustered under the second sub-cluster.

The W1 and W2 have higher support value (bootstrap value of 29) compared with the other sub-groups which only have support value of 18. Based on the morphology, all plant individuals featured similar phenotypes except AB1 (2) plant which revealed normal fruit size with small crown size and spiny leaves.

The remaining two plant individuals, traditional cultivated plant individual 3 (W3) and normal tissue cultured plant individual 1 (NTC1) were sub-clustered under the third sub-cluster with a rather high support yet low bootstrap value (32). The abnormal type 2 individual 2 [AB2 (2)] plant stay as independent with a very high support value (bootstrap value of 100).

ISSR-construct dendrogram grouped all plant genotypes into six main clusters with cophenetic correlation, r = 0.84. Cluster I, III and VI clustered two plant individuals together: Traditional cultivated plant individual 1 (W1) and abnormal type 2 individual 1 [AB2 (1)], abnormal type 1 plant individual 1 [AB1 (1)] and abnormal type 3 plant individual 3 [AB3 (3)], normal tissue cultured plant individual 1 (NTC1) and abnormal type 2 plant individual 2 [AB2 (2)] with Jaccard similarity indexes

of 0.651, 0.808 and 0.631 and were supported by a very low bootstrap value of 17, 6 and 33, respectively.

In Cluster I, the plant individual shared phenotype characteristics of normal fruit size and spiny-tip leaves. Meanwhile, in plant individuals in Cluster III shared a spiny leaf morphology whereas the plant individuals in Cluster IV revealed the normal phenotype except the small crown size shown by the AB1 (1) sample.

In clusters II and IV, they comprised of three plant individuals each. Cluster II grouped traditional cultivated plant (W2), normal tissue cultured plant (NTC2) and traditional cultivated plant (W3) with Jaccard similarity coefficient of 0.618 between the W2 and NTC2 genotypes.

The latter genotypes were supported by high yet weak clade support of 36 compared with the bootstrap value of 12 at W3 node. Meanwhile, Cluster IV grouped normal tissue cultured plant individual 3 (NTC3) and abnormal type 1 plant individual 3 [AB1 (3)] at Jaccard similarity value of 0.714.

These plant genotypes were supported by a rather high but weak clade support of 38. In contrast, the sister clade, which comprised abnormal type 3 plant individual 2 [AB3 (2)] has a bootstrap value of 15. Referring to AB1 (3) and AB3 (2) plants, both individuals shared phenotypic similarity of spiny leaf plants.

Cluster V grouped four plant individuals, namely the abnormal type 4 plant individual 1 [AB4 (1)], abnormal type 6 plant individual 1 [AB6 (1)], abnormal type 5 plant individual 2 [AB5 (2)] and abnormal type 4 plant individual 3 [AB4 (3)]. The AB4 (1) and AB6 (1) were found to have a Jaccard similarity coefficient of 0.783 and their clustering was strongly supported by high bootstrap values of 64.

Meanwhile, AB5 (2) and AB4 (3) had a very low bootstrap value of 6 and 2, respectively. Cluster V showed only plants AB4 (1) and AB4 (3) share similar morphology whereas the AB6 (1) and AB5 (2) shared similar morphology in their fruit size (small) and normal leaves. The remaining six plant individuals: Abnormal type 1 plant individual 2 [AB1 (2)], abnormal type 3 plant individual 1 [AB3 (1)], abnormal type 6 plant individual 2 [AB6 (2)], abnormal type 4 plant individual 2 [AB4 (2)], abnormal type 5 plant individual 1 [AB5 (1)] and SC were classified as independent clades.

The pooled similarity coefficient data formed UPGMA phenogram tree comprised of four main clusters with cophenetic correlation coefficient, r = 0.82. Cluster I of the dendrogram consists of six genotypes with most of the members were described as normal MD2 plant and one abnormal MD2 plant. The plant individuals which were clustered in the first cluster were traditional cultivated plant individual 1 (W1), normal tissue cultured plant individual 2 (NTC2), traditional cultivated plant individual 2 (W2), traditional cultivated plant individual 3 (W3), abnormal type 1 plant individual 1 [AB1 (1)] and normal tissue cultured plant individual 1 (NTC1).

The W1 and NTC2 were affiliated at 0.538 similarity coefficient and were supported at high bootstrap value of 35. The remaining plant genotypes; W2, W3, AB1 (1) and NTC1 were supported at bootstrap value of 32, 13, 20 and 19, respectively. On the other hand, clusters II and III clustered three plant individuals each. Cluster II, clustered normal tissue cultured plant individual 3 (NTC3), abnormal type 1 plant individual 2 [AB1 (2)] and abnormal type 2 plant individual 1 [AB2 (1)] of which NTC3 and AB1 (2) revealed 0.636 Jaccard similarity index with support value of 22.

Cluster II revealed clustering of plant samples found to have similarities of normal fruit size and normal crown size. Cluster III of the pooled data dendrogram grouped abnormal type 1 plant individual 3 [AB1 (3)], abnormal type 3 plant individual 2 [AB3 (2)] and abnormal type 3 plant individual 1 [AB3 (1)] of which AB1 (3) and AB3 (2) were related at 0.535 similarity index and supported at bootstrap value of 19. Cluster III showed the clustered plants do not share similar phenotypes.

Cluster IV of the UPGMA tree comprised all abnormal MD2 plant regenerants. The plant individuals are abnormal type 3 plant individual 3 [AB3 (3)], abnormal type 4 plant individual 1 [AB4 (1)], abnormal type 5 plant individual 2 [AB5 (2)], abnormal type 6 plant individual 1 [AB6 (1)] and abnormal type 4 plant individual 2 [AB4 (2)]. Sister clade plant individual which grouped AB3 (3) and AB4 (1) has higher support group with a bootstrap value of 23 compared to the other plant genotypes.

The remaining five plant individuals, abnormal type 6 plant individual 2 [AB6 (2)], abnormal type 4 plant individual 3 [AB4 (3)], abnormal type 5 plant individual 1 [AB5 (1)] and SC were all classified as independent clade.

The generated UPGMA phylogenetic trees confirmed the clustering patterns generated in PCA analyses whereby the phenotaxonomicals were not in agreement with the plant morphological traits. The phylogenetic tree constructed showed no clear distinction in correlating the plant's morphology with its genotype as plant samples with similar phenotypes were sorted into different RAPD and ISSR dendrograms clusters.

Similar observations were also reported in the genetic diversity study of jojoba, *Simmondsia chinensis* (Bhardwaj *et al.*, 2010), yarrow (Farajpour *et al.*, 2011), barley (Guasmi *et al.*, 2012), lentil (Seyedimoradi & Talebi, 2014), *Momordica dioica* Roxb. Ex Willd. (Rana & Das, 2015), barley (Allel *et al.*, 2017), pineapple (Wang *et al.*, 2017), *Panicum miliaceum* L. (Ghimire *et al.*, 2019), anise (Giachino, 2019), rice (Verma *et al.*, 2019), saffron *Crocus* sp. (Zarini *et al.*, 2019) and soybean (Ullah *et al.*, 2021).

The reason for the poor clustering of plant samples according to their morphology is due to the differences in the molecular marker's distribution throughout the genome and the degree of the target DNA being analysed, hence, resulting in the different classes of variation (Powell *et al.*, 1996; Russell *et al.*, 1997; Arif *et al.*, 2009; Guasmi *et al.*, 2012; Wang *et al.*, 2017; Zarini *et al.*, 2019).

The RAPD molecular marker system amplifies randomly and may represent non-coding DNA segments as well as their nature of being distributed throughout the genome may lead to the association with structurally important loci. On the other hand, the ISSR molecular marker system amplifies the region between two microsatellite loci (Zarini *et al.*, 2019). In addition, the inability to cluster plants with similar phenotype into the same group is caused by the amplification of the marker system at non-coding sequence which may confer different phenotype characteristic (Harsha *et al.*, 2016).

Since RAPD markers represent non-coding segments and distributed throughout the genome, the usage of RAPD in correlating genetic variability with morphology may lead to insignificant clustering of plant genotype with their phenotype (Harsha *et al.*, 2016). Moreover, in RAPD analysis, several reports have argued that identical mobility bands generated by RAPD molecular markers in different plant genotypes were not necessarily homologous although the bands reside with the same base pair size (Loarce *et al.*, 1996; Fernandez *et al.*, 2002; Karuppanapandian *et al.*, 2010; Rana & Das, 2016).

Furthermore, the discrepancies between RAPD and ISSR-construct dendrograms were caused by the marker sampling error and/ or the polymorphism level detected, hence, reinforcing the importance of loci number and the overall genome coverage when obtaining reliable estimates of genetic relationships among cultivars (Loarce *et al.*, 1996; Karuppanapandian *et al.*, 2010; Rana & Das, 2016; Verma *et al.*, 2017; Zarini *et al.*, 2019).

Due to this, it is important in stressing the number of band fragments produced by the marker system to generate accurate dendrogram. Previous genetic variation studies recommended that a greater number of RAPD markers be analysed to produce a RAPD-based dendrogram that faithfully reflects the genetic relationship between the genotypes (Dos Santos *et al.*, 1994; Thormann *et al.*, 1994). Apart from that, the utilisation of existing commercial DNA markers, which were developed based on genomic

DNA also results in a poor correlation between the plant genotypes as the developed DNA sequence may belong to either the transcribed regions or non-transcribed regions of the genome (Pirkhezri, 2010; Guo *et al.*, 2014).

Meanwhile, other reports on genetic diversity study of Iranian safflower (Panahi & Neghab, 2013) and primrose (*Primula heterochroma* Stapf.) recommended increasing the number of plant samples experimented on whereby they represented a successful clustering of an accession according to the geographical area by incorporating many plant accessions samples.

Another reason for the lack of success in correlating the genotypic and phenotypic data is due to the mutations caused by somaclonal variations induced during tissue culture process, which often led to an array of changes including the morphological changes among plant regenerants (Kaeppler *et al.*, 2000; Guo *et al.*, 2006; Guo *et al.*, 2007; Prakash *et al.*, 2009). In the pineapple breeding programme, the phenotypic variant was mostly induced via tissue culture propagation (Collins, 1960; Wakasa, 1977; 1979; DeWald *et al.*, 1988; Prakash *et al.*, 2009; Pérez *et al.*, 2012; Wang *et al.*, 2017).

Apparently, epigenetics, a phenomenon which alters the plant phenotypes without DNA sequence alterations is one of the primary factors in plant somaclonal variation (Boquete *et al.*, 2021). Alteration DNA methylation at cytosine residue in CpG islands in symmetric (CG or CHG, where H = A, T or G) and symmetric (CHH) is the most studied epigenetic marks. Evidence shows methylation at CG sites is often reported in plant (Boquete *et al.*, 2021; Ghosh *et al.*, 2021). This is supported in the genomic instability study on medicinal plant *Codonopsis lanceolata* (Guo *et al.*, 2006; Guo *et al.*, 2007), genome-wide study on *A. thaliana* (Cokus *et al.*, 2008) and whole genome bisulfite sequencing on mulberries (Li *et al.*, 2020).

Furthermore, environmental conditions such as abiotic (heat and salt stress) and biotic stress such as viral infections can also result in plant epigenetics. This is reported in Frey and Kürschner (2011), of which studied the effect of environmental stress in clonally raised terrestrial mosses, *Scopelophila cataractae* and *Ceratodon purpureus* via exposure to heavy metals. Apart from the epigenetic changes, the incongruence of the plant phenotypes with genomic instability possibly because the fragments of the genome amplified by the primer might not be part of phenotypic gene coding (Garcia *et al.*, 2002; Sarkosh *et al.*, 2006).

In comparison to all the constructed phylogenetic trees, only the pooled data obtained by combining two molecular markers (RAPD + ISSR) were able to cluster most normal plants in the Cluster I and abnormal plants in Cluster IV. Thus, this supports the practice of combining different molecular marker systems to increase marker sensitivity to discrete DNA differences in each plant genotypes (Giachino, 2019).

Apart from the indefinite clustering patterns among plant samples, the 1,000 bootstrap replications revealed a very low bootstrap value at the initial node of the dendrogram and relatively high bootstrap yet low support value at the subgroup level.

Generally, the bootstrap value is the statistical test to determine the stability support of the clustering in the dendrogram. The higher the bootstrap value, the higher the stability support/confidence that the clade is true (Bhardwaj *et al.*, 2010; Medhi *et al.*, 2014; Sabara & Vakharia, 2018). In line with the result obtained, the UPGMA bootstrap value generated in the genetic diversity study on jojoba (Bhardwaj *et al.*, 2010), papaya (Rodriguez *et al.*, 2010; Kanupriya *et al.*, 2012; Sabara & Vakharia, 2016) and saffron (Zarini *et al.*, 2019) reported poor/low strength support at the initial node of the clade with a significant strength support between 35 and 53.3 bootstrap value at the sub-group level.

The low bootstrap supports are the result of few characters favouring them or many characters contradicting in the data matrix (García-Sandoval, 2014). The cophenetic correlation coefficient (CCC) is applied in phenetic studies as a measure of fitness degree of a data set classification and as a criterion for the assessment of the different clustering technique efficiency

of a dendrogram (Sokal & Rohlf, 1962; Mujaju *et al.*, 2010). High cophenetic coefficient correlation (> 0.70) indicates a good fitness degree of a data set classification to summarise genetic distance/similarity matrices based on the genotype grouping (Gonçalves *et al.*, 2008; Wang *et al.*, 2017).

The coefficient correlation obtained from this study suggests a good representation between the Jaccard similarity coefficient with the generated dendrograms as the CCC r value > 0.70 (RAPD = 0.73, ISSR = 0.84, RAPD + ISSR = 0.82). This result concurs with the genetic study of sweet potato landraces (Moulin *et al.*, 2012), saffron (Zarini *et al.*, 2019), banana (*Musa* spp.) (Singh *et al.*, 2021) where the authors suggested a very high goodness fit between genetic similarity coefficient with the constructed dendrogram.

Differentiation of Population

In AMOVA, all 21 MD2 genotypes except SC were divided into eight groups comprising the traditional cultivated plant, normal tissue cultured plant and six abnormal plant types. The AMOVA result revealed a high proportion of the genetic variation exists within groups of the pineapple compared to the level of genetic variation found among the pineapple groups. The RAPD based AMOVA detected the highest genetic variation within group accounted for a 76.40% variance whereas genetic variation among group revealed a variation of 23.60%.

For ISSR analysis, molecular variation within groups estimated 72.73% variance and estimated 27.30% for molecular variance among groups meanwhile the pooled data revealed genetic variation of 74.88% within groups and 25.12% variation detected among the genotypes' group (Table 4).

A similar AMOVA result was reported in saffron where RAPD and ISSR markers revealed a high incidence of genetic variance within saffron groups (RAPD: 68%, ISSR: 64%) compared with the genetic variance detected among saffron groups (RAPD: 32%, ISSR: 36%).

The pooled data markers of RAPD and ISSR on saffron noted a 57% molecular variance within groups and 43% molecular variance among saffron groups (Zarini *et al.*, 2019). Furthermore, previous report on finger

Table 4: AMOVA based on experimented molecular markers

Source of Variation	df[a]	SS[b]	Est. var[c]	Variation (%)	PhiST[d]
RAPD					
Among groups	7	1.722	0.042	23.603	0.236
Within groups	14	1.906	0.136	76.397	
Total	21	3.627	0.178	100	
ISSR					
Among groups	7	0.503	0.014	27.274	0.273
Within groups	14	0.508	0.036	72.726	
Total	21	1.011	0.050	100	
RAPD + ISSR					
Among groups	7	0.986	0.025	25.121	0.251
Within groups	14	1.051	0.075	74.879	
Total	21	2.038	0.100	100	

The AMOVA was performed using the FAMD program with 1,000 permutations. Descriptions: (a) Degrees of freedom, (b) Sum of square, (c) Estimated variance and (d) Differentiation index

millet (Ramakrishnan *et al.*, 2015), *M. dioica* (Ras & Das, 2016) and ginger (Baruah *et al.*, 2019) were agreed with the result where higher molecular variance was detected within the groups compared to the genetic variation among the groups.

The Phi statistic in AMOVA represents the degree of differentiation between population among groups or divisions (Excoffier *et al.*, 1992). Identical to the Wright's F^{ST} and its relative G^{ST}, the Phi statistic gave value from 0 (non-differentiation or no genetic divergence) to 1 (complete differentiation between the original group and their subgroups or fixation for alternative alleles in different subpopulations (Mohammadi & Prasanna, 2003; Nybom, 2004; Jost, 2008; Laurentin; 2009; Cipriano *et al.*, 2016).

The Phi statistic generated in this study were 0.24 for RAPD marker, 0.27 for ISSR and 0.251 genetic variance for pooled data. Based on the result, the Phi statistic data presented by the independent and combination markers reflected large genetic differentiation among the MD2 genotypes. This is also reported in the genetic diversity study of *Jatropha curcas* whereby the authors indicated a large genetic differentiation occurs among *J. curcas* population as the FST value reflex index value of 0.20 (Ambrosi *et al.*, 2010).

Conversely, another study on *J. curcas* population in China revealed very high differentiation index values of between 0.29 and 0.54 (He *et al.*, 2007; Cai *et al.*, 2010). The high differentiation index values of more than 0.50 should be taken with caution as the population genotypes are so different that they could be in the process of speciation (Ovando-Medina *et al.*, 2011). Overall, the genome coverage and the total number of loci detected by the molecular markers greatly influenced the cluster analyses and AMOVA results (Lynch & Miligan, 1994). Therefore, it is important to employ good molecular markers and primer combinations to ensure sufficient polymorphic band numbers and good genome coverage.

Future Prospect

Much research on plant somaclonal variation oversees the ability of PCR-based molecular markers such as RAPD, ISSR and AFLP to cluster plants according to their phenotypic characteristics. However, applying RAPD, ISSR and combination markers to group MD2 pineapple plants according to their phenotypes is second-rate.

Hence, to improve the future somaclonal studies in clonally raised MD2 pineapple variety, it is suggested to include the mother plant in the experiment to determine any band changes, i.e., DNA sequence changes in the offspring before being planted on the field. The sources of variation can be categorised as occurring during the tissue culture process or due to environmental stress. Additionally, increasing the number of individual samples of each abnormality and the number of RAPD and ISSR molecular markers is a must to increase band number fragments and generate accurate dendrograms. Despite this, the genotypic and phenotypic of clonally raised plants are not always similar (Ghosh *et al.*, 2021) due to stress conflicting upon the plants when planted in the field, either biotic or/ and abiotic stress. These stresses would result in an epigenetic phenomenon whereby phenotypic changes are observed without changes in their DNA genome (Boquete *et al.*, 2021; Ghosh *et al.*, 2021).

Therefore, another different technique is suggested to enhance the study of genetic variability among regenerated plant populations. For example, methylation-sensitive amplification polymorphism (MSAP), a modification technique from AFLP is an appealing technique to evaluate the methylation level in the genome. The method uses methylation-sensitive restriction endonucleases like isoschizomers HpaII and Msp I to generate digestion patterns that enable the identification of methylated DNA (Yaish *et al.*, 2014).

Another suggested method is the use of transposon-based marker systems. The marker systems detect the transposable elements

distributed inside the genome of the somaclones. Sequence-specific amplified polymorphism (SSAP), inter-retrotransposon-amplified polymorphism (IRAP), retrotransposon-based insertion polymorphisms (RBIP) and retrotransposon-based insertion polymorphism (REMAP) are the examples of transposon-based markers. With the advent and advancements in next-generation sequencing (NGS) and cost-effective, high-throughput genotyping has become more convenient and accessible to researchers. Furthermore, the development of sequence characterised amplified region (SCAR) markers with higher levels of authenticity for *A. comosus* spp.

Identification should be considered by using specific primers designed from RAPD and ISSR (Ho *et al.*, 2021). The flow cytometric analysis is another useful technique to analyse genetic changes at ploidy levels and can be complemented with cytological studies such as chromosome counting (Ulvrova *et al.*, 2021). In addition, high separation techniques such as high-performance liquid chromatography (HPLC) and high-performance electrophoresis (HPCE) digest DNA to nucleotide to isolate and analyse 5-mC (Miguel & Marum, 2011).

The separation technique is recommended due to high specificity and sensitivity; however, the method is time-consuming (Berdasco *et al.*, 2009). The application of NGS has led to remarkable advances to revolutionise plant genotyping and breeding. Single-nucleotide polymorphism (SNP) based genotyping is another preferable method for studying genetic variation in *in vitro* plants. The method offers several advantages, including cost-effectiveness, timesaving, genome-wide coverage, high resolution and the establishment of syntenic relationships.

Moreover, SNP-based genotyping is proven in genotyping screening, genetic mapping, purity testing, parent testing, haplotype map construction, association mapping, marker-assisted selection (MAS) and genomic selection (GS).

Conclusion

The assessment using DNA molecular markers reveals plant genotypes were not in agreement with the plant's morphological traits despite the informativeness showed by both RAPD and ISSR markers. The inability for the genotype-phenotype correlation of the studied markers is due to different genome coverage of the markers and the number of loci detected in the plant genome as well as DNA methylation that was triggered by tissue culture processes.

Disregarding the inability of genotype-phenotype correlation by the studied markers, the markers showed high proportion of genetic differentiation detected within the groups and reflected large genetic differentiation among the MD2 genotypes through AMOVA and Phi statistics, respectively.

In general, the detected number of loci and genome coverage in addition to the increase of biological replicates are important in obtaining good quality data for better analysis. Therefore, the findings from this study provides new information for future studies on genetic variation in tissue culture regenerants especially among clonally raised MD2 pineapples and other pineapple varieties.

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