COMPARATIVE ANALYSIS OF DAMD, ISSR, AND SCOT MOLECULAR MARKERS ON CRYOPRESERVED *LUDISIA DISCOLOR* AXILLARY BUDS

Kirutika Selva Rajan[1a], Hazirah Burkhan[2a], Bee Lynn Chew[3a], Suganthi Appalasamy[4b], Ranjetta Poobathy[5c], and Sreeramanan Subramaniam[5a,d,e,f*]

[a] School of Biological Sciences, Universiti Sains Malaysia, 11800 Georgetown, Penang, MALAYSIA. Email: kirutika28@gmail.com[1], xiraburkhan@yahoo.com[2], beelynnchew@usm.my[3], sreeramanan@usm.my[6]
[b]Department of Natural Resource and Sustainability, Faculty of Earth Science, Universiti Malaysia Kelantan, Jeli Campus, Locked Bag No. 100, 17600 Jeli, Kelantan, MALAYSIA. Email: suganthi.a@umk.edu.my[4]
[c]School of Biological Sciences, Quest International University, 30250, Ipoh, Perak, Malaysia. Email: ranjetta.poobathy@qiup.edu.my[5]
[d]National Poison Centre, Universiti Sains Malaysia, 11800 Georgetown, Penang, MALAYSIA. Email: sreeramanan@usm.my[6]
[e]School of Chemical Engineering Technology, Universiti Malaysia Perlis, 02600 Arau, Perlis, MALAYSIA. Email: sreeramanan@usm.my[6]
[f]Centre for Chemical Biology, Universiti Sains Malaysia, 11900, Bayan Lepas, Penang, MALAYSIA. Email: sreeramanan@usm.my[6]
*Corresponding Author: sreeramanan@usm.my / sreeramanan@gmail.com
Received: 17th Nov 2021 Accepted: 14th Feb 2022 Published: 30th Jun 2022
DOI: https://doi.org/10.22452/mjs.vol41no2.1

ABSTRACT The jewel orchid *Ludisia discolor* is the only species found under its genus (*Ludisia*). This wild orchid is well known for its striking foliage and its medicinal benefits. Since the population of this valuable species is becoming scarce, protecting it is crucial. Cryopreservation is a practicable long-term germplasm conservation approach using the V cryo-plate method, which was developed using *L. discolor* axillary buds under optimum conditions. This study aims to carry out a comparative analysis of DAMD, ISSR, and SCOT molecular markers on cryopreserved *L. discolor* axillary buds. For the screening of the somaclonal variation, 3 week-old treated (cryopreserved and non-cryopreserved) explants were utilized in comparison with the stock cultures used as a control. The genetic stability was assessed using a total of 20 DAMD, 20 ISSR, and 10 SCoT molecular markers. In this study, the somaclonal variations detected in cryopreserved explant using DAMD, ISSR, and SCoT molecular markers were 12.5, 4.17 and 10.53%, respectively. However, the somaclonal variations detected in non-cryopreserved explants using DAMD, ISSR, and SCoT were 6.15, 5.77 and 10%, respectively. Thus, DAMD and SCoT were chosen as better markers for genetic screening as compared to ISSR.

Keywords*:* cryopreservation, *Ludisia discolor*, molecular analysis, DAMD, ISSR, SCoT

1. INTRODUCTION

The Orchidaceae is the biggest family in flowering plants, and it consists of approximately 20,000 orchids (Moreira and Isaias, 2008). Besides being cultivated for ornamental purposes, orchids are also widely believed to have medicinal values. Orchids are one of Malaysia's most popular cut flowers, with an estimated annual value of MYR 150 million (Ibrahim *et al*., 2012). *L. discolour*, also known as "Jewel orchid', is an endangered terrestrial orchid species native to Southeast Asia, which can be found in Malaysia (Poobathy *et al*., 2019). *L. discolor* is renowned for its captivating leaves with shiny and intrinsic reticulations. The orchid is a perennial herb that blooms every year from October to December (Tseng et al., 2006). After two to three years of growth, the plantlets mature and multiply through seed production. Wild *L. discolor* which are commonly propagated through seeds have low germination rates and are currently threatened with extinction due to overharvesting (Ket *et al*., 2004; Lim *et al*., 2013).

Plant cryopreservation technology is a reliable *ex-situ* conservation method. It has been marked as an important technique for genetic conservation of endangered, hybrids, unique and rare orchid species for future selection and breeding purposes (Thammasiri, 2005). Cryopreservation is an ideal long-term germplasm conservation technique because it is time-saving, requires minimum space, cost, and labour (Suzuki *et al*., 2008; Engelmann, 2011).

Through cryopreservation, the metabolism of the target genetic resource is significantly reduced by slowing down all biochemical processes and biological deterioration at -196°C (Engelmann, 2011). Yamamoto *et al.* (2011) invented the V cryo-plate method, a fusion of encapsulation-dehydration and droplet-vitrification methods. The perks of utilizing the V cryo-plate method include its simplicity, rapid cooling and warming rate and low risk of explant injury, which eventually results in a high recovery percentage (Niino *et al*., 2013; Salma *et al*., 2014; Matsumo *et al*., 2015). A full set of V cryo-plate protocol involves preculture of excised explants on solidified medium with optimised sucrose concentration for a specified duration at room temperature (RT), osmoprotectant in (LS), PVS2, rapid cooling and warming of samples in a water bath, rewarming (RS) and finally, regeneration *in vitro*. Preliminary viability assessments were conducted using the 2,3,5-triphenyltetrazolium chloride (TTC) assay (Whiters, 1985). The benefits of TTC assay include ease of performance, repeatability and the lack of requirement for sophisticated equipment (Mikula *et al*., 2006).

Cryopreservation can cause genetic variation in cryopreserved explants (Harding, 2004). Variation at the molecular level should be omitted to achieve a true-to-type plant. Genetic variation can be determined by utilizing molecular markers. Directed amplification of minisatellite DNA (DAMD), inter simple sequence repeats (ISSR), and start codon targeted (SCoT) are the markers that can be used for molecular studies in cryopreservation. DAMD and ISSR markers are very useful tools for hereditary diversity studies in plants. They exhibit a comprehensive depiction of the degree of diversity (Purayil *et al*., 2018). On the other hand, the SCoT marker is widely used in the study of phylogenetics, which has been proven to produce better resolvability than other markers (Etminan *et al*., 2016).

The goal of this research was to determine the genetic stability of cryo-treated explants preserved using the V cryo-plate method. In comparison with the control, genetic variation of treated explants was tested using DAMD, ISSR, and SCoT molecular markers.

2. MATERIALS AND METHODS

2.1 Plant material

Micropropagated axillary buds (3-5 mm) were used as plant material for cryopreservation study (Figure 1). Based on the preliminary tests, it was found that the most effective media for *L. discolor* micropropagation was half-strength MS media added with 30 g/L sucrose, 1 mg/L 1-Naphthaleneacetic acid (NAA), 0.1 mg/L Thidiazuron (TDZ), and 3.5 g/L Gelrite (DUCHEFA, Netherlands). Before autoclaving the media at 121 ℃ for 15 minutes, the pH was adjusted to 5.7. The cultures were maintained at 25 ℃ for 16 hours photoperiod (PhilipsTLD, 36 W, 150 $mol.m^{-2}.s^{-1}$).

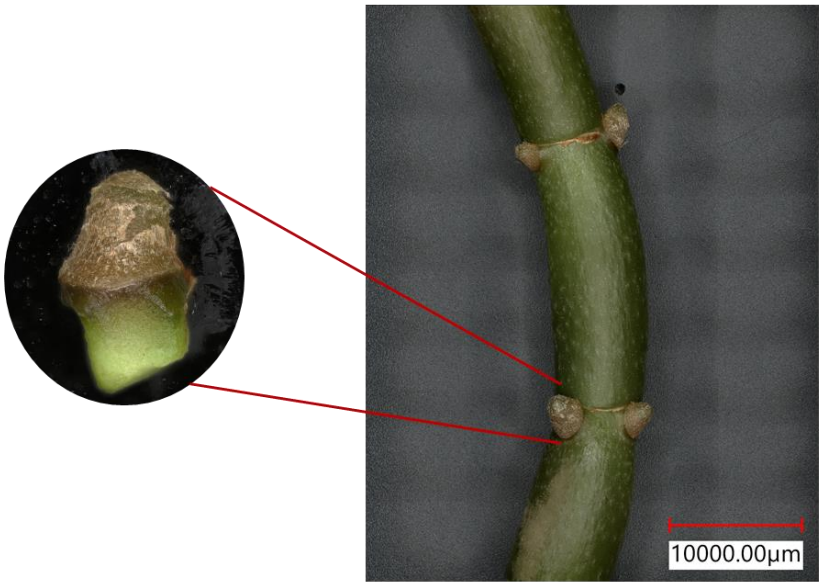

Figure 1. Axillary bud used for cryopreservation. Scale bar represents 10000 µm.

2.3 DNA extraction

DNA extraction of axillary buds from three different conditions, i.e., stock axillary buds and treated axillary buds (cryopreserved and non-cryopreserved) was used to examine the genetic stability. The control (stock axillary bud) was obtained from 3 month-old mother plant. Whereas, the treated axillary buds were obtained 3 weeks after the V cryo-plate cryopreservation process. DNA extraction was done according to the protocol of the Wizard® Genomic DNA Purification Kit (Promega, USA). Axillary buds weighing 100 mg from three conditions were ground using mortar and pestle to extract the DNA.

2.4 PCR amplification using DNA markers

The genetic stability of treated *L. discolor* was screened by using a total of 20 DAMD, 20 ISSR, and 10 SCoT primers. All primers were acquired from 1st Base Malaysia. PCR amplification was done according to Amirah et al. (2019). In a 200 µL reaction mixture, 12.5 µL of exTEN 2X PCR Mastermix (Base Asia), 5 µL of 0.2 µM primer, 3 µL of 20 ng/µL template DNA, and 7 µL of double distilled water were mixed using the pipette. The PCR amplification was conducted using MyCycler™ Thermal Cycler. The initial denaturation parameters for PCR

amplification were programmed at 95 °C for 4 minutes, followed by denaturation at 95 °C for 30 seconds, annealing temperature for 30 seconds, an extension at 72 °C for 1 minute, and a cycle of final extension at 72 °C for 10 minutes.

2.5 Agarose gel electrophoresis

Electrophoresis was done on amplified PCR products using molten 1.5% (w/v) agarose solvated in 40 mL 1xTris-Borate-EDTA (TBE). The mixture was microwaved until it came to a boil. It was left to cool down for some time. The mixture was stained with 2 µL of redsafe stain (RedSafe™ Nucleic Acid Staining Solution (20,000x**)**, iNtRON, South Korea). Finally, the liquid was transferred into a Mini Gel Caster and allowed to firm for 30 minutes at room temperature. Then, the caster was transferred into an agarose gel electrophoresis system base filled with 0.5× TBE buffer. To run electrophoresis, the wells in agarose gel were loaded with 6 µL of 1 kb ladder, followed by 6 µL of 100 bp ladder and 6 µL of amplified PCR products. The agarose gels were electrophoresed for 75 minutes at 75V.

2.6 Determination of polymorphisms analysis

Similarity indices (SI) comparison was carried out between control axillary buds and treated (cryopreserved & non-cryopreserved) axillary buds. DNA fragment patterns were determined by these SI. A score of 1 was given for any presence of the band, while 0 was given in the absence of the band. The SI was calculated according to the following formula (Asnita and Norzulaani, 2006).

$$\textbf{Similarity index (Si)} = \frac{2Nxy}{(Nx+Ny)}$$

where **Nxy** = number of monomorphic bands in between the control and treated (cryopreserved/ non-cryopreserved) groups
Nx = total number of bands present in the control group
Ny = total number of bands present in the treated (cryopreserved/ non-cryopreserved) group

3. RESULTS

3.1 DAMD analysis of treated axillary buds in comparison with control axillary buds

Molecular analysis using the DAMD marker was carried out to assess and compare the genetic stability of cryopreserved and non-cryopreserved axillary buds in comparison with control. Out of 25 primers used, 20 produced coherent and precise bands. A total of 71 bands were obtained from control axillary buds, 65 from non-cryopreserved and 64 from cryopreserved axillary buds (Figure 2). The range of amplification from the products was between 100 to 1500 bp.

The genetic stability comparison between non-cryopreserved and control axillary buds showed that out of 65 bands obtained from non-cryopreserved axillary buds, 61 were monomorphic and 4 polymorphic. The four polymorphic bands produced were from the primers URP6R, URP30F, HBV5 and HBV3, resulting in a 6.15% polymorphic percentage with an SI index between 0.5 and 0.8.

On the other hand, genetic stability assessment between cryopreserved and control axillary buds showed that a total of 64 bands were generated from the cryopreserved sample. Out of the 64 bands, 56 were monomorphic and 8 were polymorphic. The polymorphism in cryopreserved axillary buds was derived from primers URP30F (2), HBV5 (1), HBV3 (2), HVR (1), INS (1) and HVV (1).

The DAMD primer demonstrated 12.5% of polymorphism and 87.5% of monomorphism in cryopreserved axillary buds. The DAMD DNA fragments sizes ranged from 100 to 1500 bp.

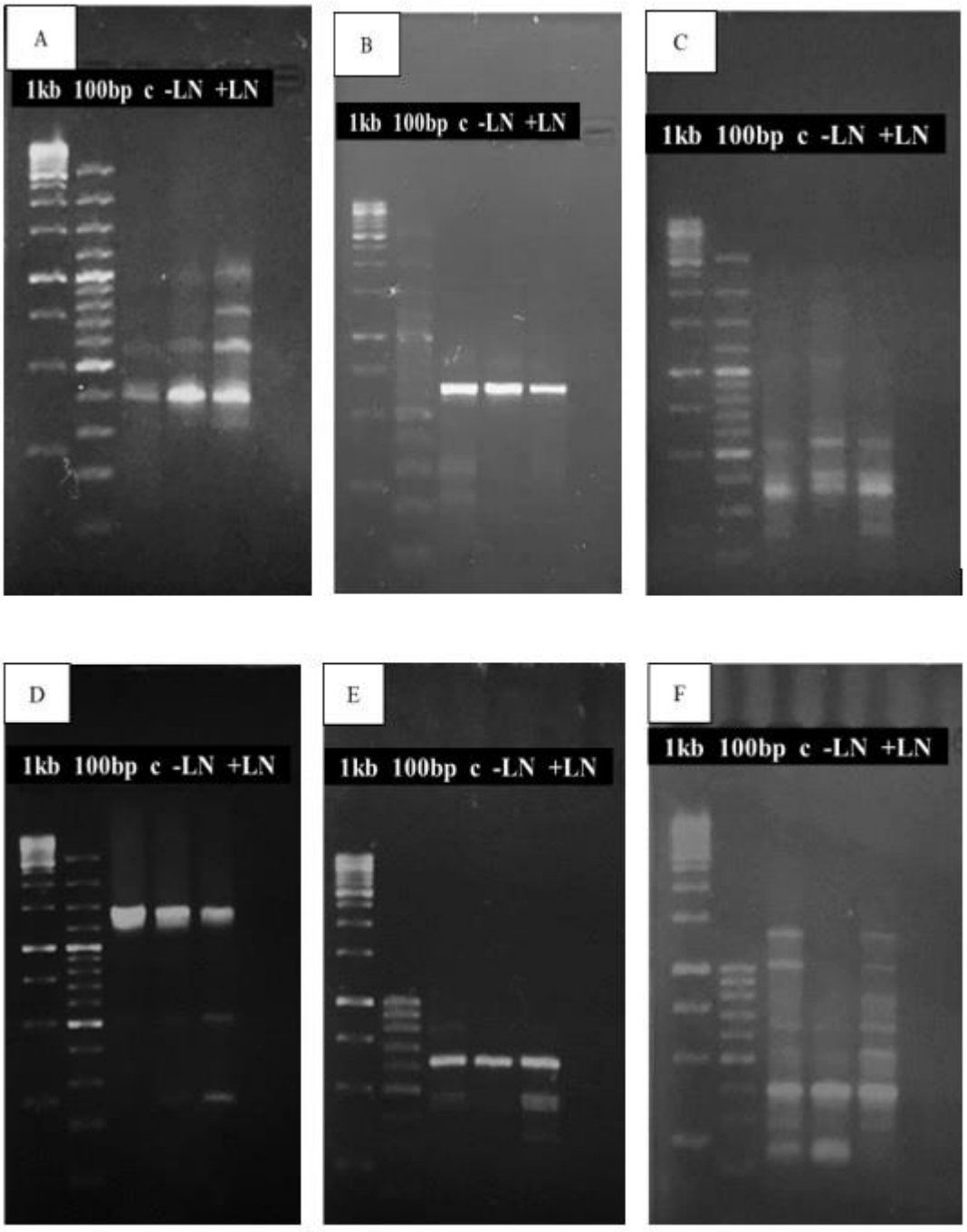

Figure 2. Banding profile of cryopreserved axillary buds (+LN) and non-cryopreserved axillary buds (-LN) as compared with stock axillary buds (c) using DAMD primers (A) URP30F; (B) URP32F; (C) URP38F; (D) HBV3; (E) HVR; and (F) INS. Molecular ladders = 1kb & 100bp

3.2 ISSR analysis of cryopreserved and non-cryopreserved axillary buds as compared with untreated control axillary buds

ISSR molecular marker was utilised to determine the genetic variance in the treated axillary buds as compared with control axillary buds. From the total of 23 ISSR primers, only 20 produced visible bands (Figure 3). Overall, 20 primers produced 68 bands in control, 54 in cryopreserved, and 54 in non-cryopreserved axillary buds. The similarity index (SI) for treated axillary buds ranged from 0.4 to 1. The ISSR band observed was either entirely monomorphic or polymorphic as compared with the control. A score of 1.0 was given for each monomorphic band while a score of less than 1.0 was given for the polymorphic band. The amplicons ranged from 100 to 900 bp.

Out of 52 bands from non-cryopreserved axillary buds, 49 bands were monomorphic. In comparison, 3 bands represented polymorphism (N2, N3, and N10) as compared with that of control axillary buds. Polymorphism was demonstrated by the presence of an extra or reduced number of bands. The percentage of polymorphism was 5.77%, while the percentage of monomorphism was 94.23%. Only 10 out of the total of 20 primers scored an SI value of 1.0. Meanwhile, the remaining 10 primers produced SI values ranging from 0.4 to 0.91. A total of 46 bands were monomorphic and 2 others were polymorphic. The percentage of polymorphism was 4.17%, with a large percentage of 95.83% representing monomorphism. It was observed that 10 primers demonstrated an SI value of 1.0. The samples yielded DNA fragments ranging in size from 100 to 900 bp.

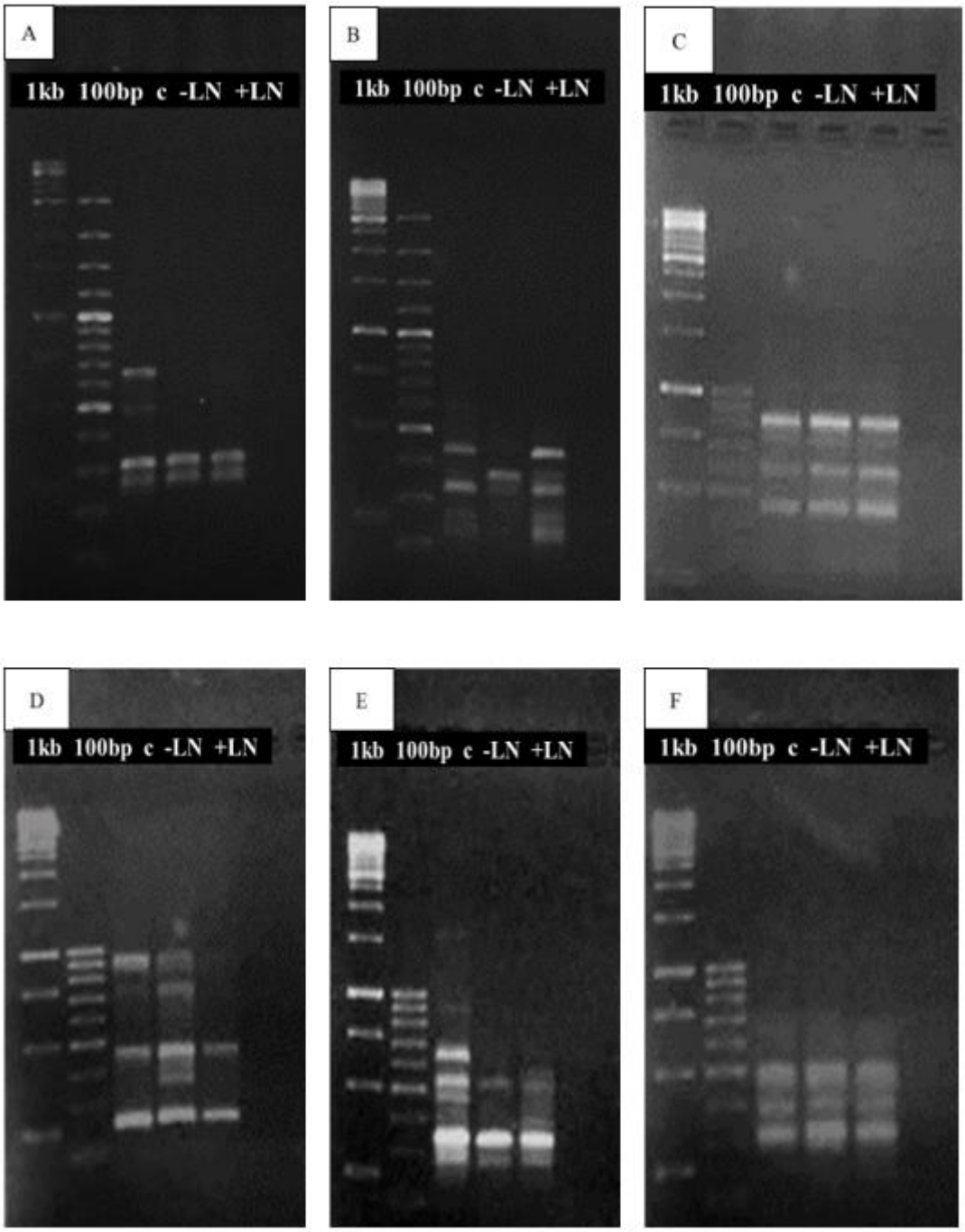

Figure 3. Banding profile of cryopreserved axillary buds (+LN) and non-cryopreserved axillary buds (-LN) as compared with stock axillary buds (c) using ISSR primers (A) N7; (B) N8; (C) N10; (D) N11; (E) H12; and (F) U807 . Molecular ladders = 1kb & 100bp

3.3 SCoT analysis of cryopreserved and non-cryopreserved axillary buds as compared with untreated control axillary buds

Molecular analysis using the SCoT marker was carried out to assess and compare the genetic stability of cryopreserved and non-cryopreserved axillary buds in comparison with control axillary buds. From a total of 12 primers, 10 primers generated reproducible and precise bands resulting in 40 bands obtained from control, 40 bands from non-cryopreserved and 38 bands from cryopreserved axillary buds (Figure 4). The amplification products were in the range of 300 to 450bp.

Comparison of genetic stability between non-cryopreserved and control axillary buds demonstrated that out of 40 bands obtained from non-cryopreserved axillary buds, 36 were monomorphic and 4 were polymorphic. The four polymorphic bands produced were derived from the primers S1, S2, and S_9A, which contributed to a 10% polymorphic percentage with an SI index between 0.67 and 0.89. The sizes of DNA fragments generated from the sample varied from 100 to 1000bp.

On the other hand, a comparison of genetic stability between cryopreserved axillary buds and control axillary buds showed that out of 38 bands generated from the cryopreserved axillary buds, 34 were monomorphic and 4 were polymorphic. The polymorphism in cryopreserved axillary buds was derived from primers S2, S7, S_9A, and S11. The SCoT profile was represented with 10.53% of polymorphism and 89.47% of monomorphism. The size of the DNA bands from the samples ranged from 100 to 2500 bp.

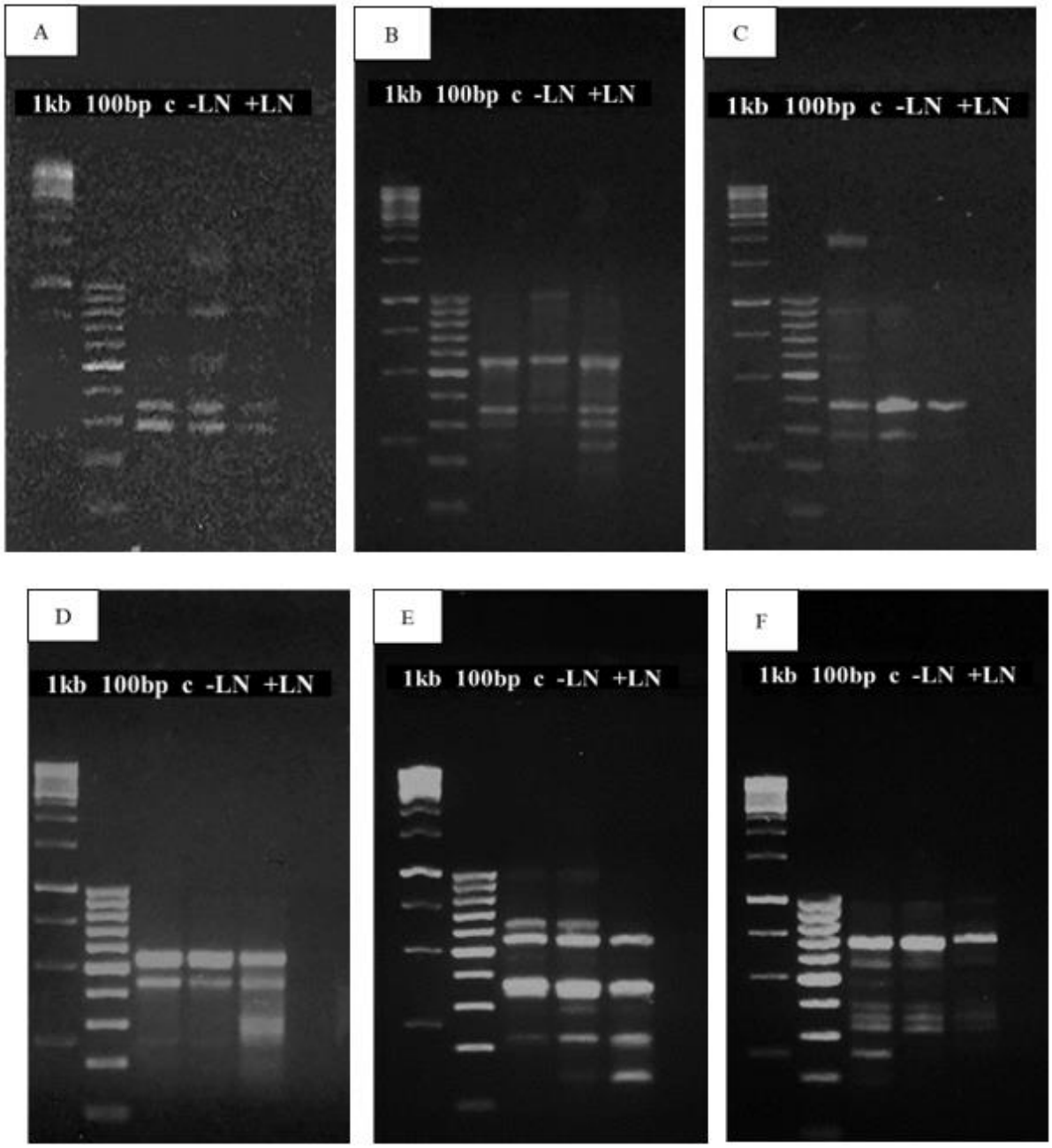

Figure 4. Banding profile of cryopreserved axillary buds (+LN) and non-cryopreserved axillary buds (-LN) as compared with stock axillary buds (c) using SCoT primers. (A) S1; (B) S2; (C) S4; (D) S7; (E) S9_A; and (F) S9_B. Molecular ladders = 1kb & 100bp

Molecular markers used in this study were DAMD, ISSR, and SCoT. The aim of using molecular markers in this study was to detect the presence of possible polymorphisms in cryopreserved and non-cryopreserved axillary buds in comparison with control axillary buds. The total number of polymorphic bands observed were 8 out of 64 DAMD marker bands, 4 out of 40 ISSR marker bands, and 4 out of 38 SCoT marker bands (Table 1). The percentage of polymorphic bands in cryopreserved samples as detected by DAMD, ISSR, and SCoT were 12.5, 4.17, and 10.53, respectively. In addition, for non-cryopreserved axillary buds, DAMD

produced a higher number of bands (65 bands) as compared with ISSR (40 bands) and SCoT (40 bands). The percentage of polymorphic bands obtained by using DAMD was 6.15%, which was higher than that of ISSR and SCoT, with 5.77 and 10% of polymorphism, respectively (Table 2). Thus, the DAMD marker was regarded as more sensitive in detecting polymorphism in cryopreserved axillary buds while the SCoT marker was found as more accurate than DAMD and ISSR in detecting polymorphism in non-cryopreserved axillary buds.

Table 1: Comparative analysis of DAMD, ISSR, and SCoT markers for cryopreserved axillary buds

Details of the primers	**DAMD**	**ISSR**	**SCoT**
Number of primers selected	20	20	10
Amount of bands produced	64	48	38
Amount of polymorphic bands produced	8	2	4
Polymorphism percentage	12.5	4.17	10.53
Number of bands/primers (Average)	3.2	2.4	3.8
Number of polymorphic bands/primers (Average)	0.4	0.1	0.4

Table 2: Comparative analysis of DAMD, ISSR, and SCoT markers for non-cryopreserved axillary buds

Details of the primers	**DAMD**	**ISSR**	**SCoT**
Number of primers selected	20	20	10
Number of bands produced	65	52	40
Number of polymorphic bands produced	4	3	4
Polymorphism percentage	6.15	5.77	10
Number of bands/primers (Average)	3.25	2.6	4
Number of polymorphic bands/primers (Average)	0.2	0.15	0.4

4. DISCUSSION

The main purpose of cryopreservation is to develop efficient long-term conservation of genetic material. The survival of plant tissue and its ability to regenerate into a whole plant defines the success rate of cryopreservation. Multiple treatments carried out during cryopreservation, such as stress-yielding dehydration and freezing might affect genetic stability. PCR-based molecular markers are used to observe the variance (Harding, 2004). In this study, three types of PCR-based markers were selected and used for comparison due to their productivity and simplicity. Detecting genetic variance with more than one marker yields accurate results in terms of genetic integrity analyses as different markers detect polymorphism in different regions of a genome (Palombi & Damiano, 2002).

Under stringent conditions, DAMD is a reliable and duplicatable marker for genetic analysis. During PCR, the DAMD minisatellite sequences are employed as a single primer for directing the amplification at many different sections of a genome. (Heath *et al*., 1993). Other than being used to detect polymorphism, molecular markers are also utilised to classify the genetic resources of the plants for identification of collections, analysis of genetic diversity

and determination of taxonomic relationships (Kameswara, 2004). DAMD marker has also been reported to detect a high degree of polymorphism. Previously, Jain *et al*. (2017) reported that DAMD marker was used to study the diversity of *Sechium edule* collections from several areas in India, whereby high genetic variations were detected.

On the other hand, ISSR primers are broadly distributed throughout the DNA genome resulting in a high degree of DNA amplification, resulting in a large number of fragments generated per primer. ISSR is capable of identifying variation in the regions in between microsatellites (Yin *et al*., 2013). According to Rawat *et al*. (2013), ISSR demonstrated high reproducibility via genetic variation study at inter and intraspecific levels (Thakur *et al.,* 2020). Uchoi *et al*. (2017) observed genetic variance using ISSR marker in *Citrus medica* L. cultivars from different regions in India, whereby polymorphism was detected. In short, DAMD and ISSR are efficient in detecting genetic homogeneity, genetic diversity and species evolution (Seyedimoradi *et al*., 2012).

SCoT marker is a unique, simple, and reliable approach, which depends on the translation of the start codon. SCoT has been utilized for genetic evaluation in various plant species, such as rice (Collard and Mackill, 2009; Samarina *et al*., 2021), peanut (Xiong *et al*., 2009, 2010, 2011), and potato (Gorji *et al*., 2011). SCoT, a single primer amplification molecular marker, is an easy, time-saving and energy-efficient technique.

This study reported polymorphism in both cryopreserved and non-cryopreserved *L. discolor* using DAMD, ISSR, and SCoT markers. The results demonstrate that the cryopreservation treatment can result in genetic variation. According to Adu-Gyamfi *et al*. (2016), propagation of plant material *in vitro* can result in a significant rise in the frequency of variations, which can be genetic or epigenetic. Few factors that have been associated with these variations that result from plant propagation *in vitro* include medium composition, the origin of donor tissue, number of regeneration events and propagation technique used.

Moreover, cryopreservation techniques used for long-term germplasm conservation involve multiple procedures including rapid and controlled cooling, treatment with chemical cryoprotective solutions and exposure of tissues to physical, chemical and physiological stresses, all of which may lead to irreversible cryoinjury. The impact of cryoinjury at the genomic level of an organism remains unknown. Due to the multiple procedures in the cryopreservation technique, the surviving plants may still be vulnerable to the effects of somaclonal variation and may eventually exhibit changes in their genotype or phenotype profiles. Thus, before storing the plant germplasm, it is critical to determine that the plant is genetically similar to the mother plant (Harding, 2004).

The low frequency of polymorphic bands suggests that the cryopreserved and non-cryopreserved axillary buds have a low genetic base. Different DNA markers can be used in the future to confirm the polymorphism rate in cryopreserved axillary buds obtained from the DAMD, ISSR, and SCoT analyses. Further evaluation of somaclonal variation based on morphological, physiological, and biochemical characteristics needs to be determined for the long-term preservation of cryopreserved axillary buds of *L. discolor*.

5. CONCLUSION

This study has shown that the DAMD marker is more sensitive and precise than SCoT and ISSR markers for the detection of polymorphism in the cryopreserved axillary buds of *L. discolor*. However, in comparison with the conventional dominant markers of DAMD and ISSR, gene-targeted SCoT markers were found most suitable for the detection of polymorphism in non-cryopreserved axillary buds.

6. ACKNOWLEDGMENT

The authors would like to acknowledge the financial support from the Malaysian Ministry of Higher Education via the FRGS 2019 grant (FRGS/1/2018/STG03/USM/02/5) and the Agricultural Crop Trust. The authors also express gratitude to Universiti Sains Malaysia (USM) for supporting this study.

Author contribution RKS, ASM designed and carried out the experiments. RKS and SS analysed the data and wrote the manuscript. SS internalized and supervised the research. BLC, PR, and AS supervised the research. RKS and BH performed molecular analysis and verify the band pattern and comparative analysis data. The manuscript was revised and edited by SS.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that there are no conflicts of interest.

7. REFERENCES

Adu-Gyamfi R., Wetten A and Marcelino Rodríguez López C.(2016) Effect of cryopreservation and post-cryopreservation somatic embryogenesis on the epigenetic fidelity of cocoa (*Theobroma cacao* L.). *PLoS One*, 11(7): 1-13.

Amirah Z J., Elexson N., Grace B and Diyana Z.(2019) Detection of cholera toxin (ctxA and ctxAB) genes in *Vibrio cholerae* isolated from clinical and environmental samples in Limbang Sarawak by multiplex polymerase chain reaction (PCR). *International Postgraduate Symposium in Biotechnology*, 2019. 18.

Asnita A H and Norzulaani K.(2006) Direct regeneration and RAPD assessment of male inflorescence derived plants of *Musa acuminata* cv. *Berangan. Asia Pacific Journal of Molecular Biology and Biotechnology*, 14(1): 11-17.

Benson E E.(2008) Cryopreservation of phytodiversity: a critical appraisal of theory & practice. *Critical reviews in Plant Sciences*, 27(3): 141-219.

Collard B C Y and Mackill D J.(2009) Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. *Plant Molecular Biology Reporter*, 27(1): 86-93.

Engelmann F.(2011) Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology-Plant*, 47(1): 5-16.

Etminan A., Pour-Aboughadareh A., Mohammadi R., Ahmadi-Rad A., Noori A., Mahdavian Z and Moradi Z.(2016) Applicability of start codon targeted (SCoT) and

inter-simple sequence repeat (ISSR) markers for genetic diversity analysis in durum wheat genotypes. *Biotechnology and Biotechnological Equipment* 30(6): 1075–1081.

Gorji A M., Poczai P., Polgar Z and Taller J.(2011) Efficiency of arbitrarily amplified dominant markers (SCoT, ISSR and RAPD) for diagnostic fingerprinting in tetraploid potato. *American Journal of Potato Research,* 88(3): 226–237.

Harding K.(2004) Genetic integrity of cryopreserved plant cells: a review. *CryoLetters,* 25(1): 3-22.

Heath D F., Wei Z., Fowler W K and Nelson V W.(1993) Comparison of spectral radiance calibrations of SBUV-2 satellite ozone monitoring instruments using integrating sphere and flat-plate diffuser techniques. *Metrologia,* 30(4): 259.

Ibrahim B., Sowemimo A., Van Rooyen A and Van De Venter M.(2012) Anti-inflammatory, analgesic and antioxidant activities of *Cyathula prostrata* (L.) Blume (Amaranthaceae). *Journal of Ethnopharmacol.* 141(1): 282–289.

Jain J R., Timsina B., Satyan K B and Manohar S H.(2017) A comparative assessment of morphological and molecular diversity among *Sechium edule* (Jacq.) Sw. accessions in India. *3 Biotech,* 7(2): 1-8.

Kameswara R N.(2004) Plant genetic resources: advancing conservation and use through biotechnology. *African Journal of Biotechnology* 3(2): 136–145.

Ket N V., Hahn E J., Park S Y., Chakrabarty D and Paek K Y.(2004) Micropropagation of an endangered orchid *Anoectochilus formosanus*. *Biologia Plantarum*, 48(3): 339-344.

Lim C L., Jutta M., Kiew R., O'Byrne P and Ong P T.(2013) Conservation status of some orchids in Peninsular Malaysia. *Malesian Orchid* Journal, 12: 133-146.

Matsumoto T., Yamamoto S I., Fukui K., Rafique T., Engelmann F and Niino T.(2015) Cryopreservation of persimmon shoot tips from dormant buds using the D cryo-plate technique. *The Horticulture Journal*, MI-043.

Mikuła A., Niedzielski M and Rybczyski J J.(2006) The use of TTC reduction assay for assessment of *Gentiana* spp. cell suspension viability after cryopreservation. *Acta Physiologiae Plantarum*, 28(4): 315-324.

Moreira A S F P and Isaias R M D S.(2008) Comparative anatomy of the absorption roots of terrestrial and epiphytic orchids. *Brazilian Archives of Biology and Technology*, 51(1): 83-93.

Niino, T., Yamamoto, S., Fukui, K., Martínez, C.R., Arizaga M.V., Matsumoto, T., & Engelmann, F.(2013) Dehydration improves cryopreservation of mat rush (*Juncus decipiens* Nakai) basal stem buds on cryo-plates. *CryoLetters* 34(6): 549– 560.

Palombi M and Damiano C.(2002) Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). *Plant Cell Reports,* 20(11): 1061-1066.

Poobathy R., Zakaria R., Murugaiyah V and Subramaniam S.(2019) Surface sterilization and micropropagation of *Ludisia discolor*. *Biocatalysis and Agricultural Biotechnology*, 22: 101380.

Purayil F T., Robert G A., Gothandam K M., Kurup S., Subramaniam S and Cheruth A J.(2018) Genetic variability in selected date palm (*Phoenix dactylifera* L.) cultivars of United Arab Emirates using ISSR and DAMD markers. *3 Biotech*, 8(2): 109.

Rawat J M., Rawat B., Mehrotra S., Chandra A and Nautiyal, S.(2013) ISSR and RAPD based evaluation of genetic fidelity and active ingredient analysis of regenerated plants of *Picrorhiza kurroa*. *Acta Physiologiae Plantarum,* 35(6): 1797-1805.

Salma M., Fki L., Engelmann-Sylvestre I., Niino T and Engelmann F.(2014) Comparison of droplet-vitrification and D-cryoplate for cryopreservation of date palm (*Phoenix dactylifera* L.) polyembryonic masses. *Scientia Horticulturae*, 179: 91-97.

Samarina L S., Malyarovskaya V I., Reim S., Yakushina L G., Koninskaya N G., Klemeshova K V., Shkhalakhov R M., Matskiv A O, Shurkina E S., Slepchenko N A., Ryndin A V and Gabueva, T. Y.(2021) Transferability of ISSR, SCoT and SSR markers for *Chrysanthemum*× *Morifolium Ramat* and genetic relationships among commercial Russian cultivars. *Plants 2021*, 10, 1302.

Seyedimoradi H., Talebi R., Hassani D and Karami F.(2012) Comparative genetic diversity analysis in Iranian local grapevine cultivars using ISSR and DAMD molecular markers. *Environmental and Experimental Biology*, 10: 125-132.

Suzuki M., Tandon P., Ishikawa M and Toyomasu, T.(2008). Development of a new vitrification solution, VSL, and its application to the cryopreservation of gentian axillary buds. *Plant Biotechnology Reports*, 2(2): 123-131.

Thakur M., Sharma V and Chauhan A.(2020) Genetic fidelity assessment of long term *in vitro* shoot cultures and regenerated plants in Japanese plum cvs Santa Rosa and Frontier through RAPD, ISSR and SCoT markers. *South African Journal of Botany*, 22(1):1-6.

Thammasiri K.(2005) Conservation and sustainable development of orchids in Thailand. In Proceedings of the 17th World Orchid Conference: Sustaining Orchids for the Future 2002. Natural History Publications (Borneo) Sdn Bhd, pp. 211-220.

Tseng C., Shang H F., Wang L F., Su B., Hsu C., Kao H Y., Cheng K T.(2006) Antitumor and immune stimulating effects of *Anoectochilus formosanus* Hayata.

Phytomedicine, 13(5): 366-370.

Uchoi A., Malik S K., Choudhary R., Kumar S., Pal D., Rohini M R and Chaudhury R.(2017) Molecular markers in assessing genetic variation of Indian citron (*Citrus medica* L.) cultivars collected from different parts of India. *Indian Journal of Biotechnology*, 16(3): 346-356.

Whiters L A.(1985) Cryopreservation of cultured plant cells and protoplasts. *Cell culture and somatic cell genetics of plants*. 2: 253-316.

Xiong F Q., Tang R H., Chen Z L., Pan L H and Zhuang W J.(2009) SCoT: a novel gene targeted marker technique based on the translation start codon *Molecular Plant Breeding.* 7(3): 635–638.

Xiong F Q., Jiang J., Zhong R C., Han Z Q., He L Q., Li Z and Tang R H.(2010) Application of SCoT molecular marker in genus *Arachis Acta Agronomica Sinica.* 36(12): 2055-2061.

Xiong F., Zhong R., Han Z., Jiang J., He L., Zhuang W and Tang R.(2011) Start codon targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated peanut (*Arachis hypogaea* L.) genotypes *Molecular Biology Reports.* 38(5): 3487-3494.

Yamamoto S I., Rafique T., Priyantha W S., Fukui K., Matsumoto T and Niino T.(2011) Development of a cryopreservation procedure using aluminium cryo plates. *CryoLetters* 32(3): 256-265.

Yin Z F., Zhao B., Bi W L., Chen L and Wang Q C.(2013) Direct shoot regeneration from basal leaf segments of *Lilium* and assessment of genetic stability in regenerants by ISSR and AFLP markers *In Vitro Cellular & Developmental Biology-Plant.* 49(3): 333-342.