Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i3.3004

Research Article

DAMD And ISSR DNA Molecular Analyses of Regenerated Cryopreserved *Dendrobium* Sabin Blue's Protocorm-Like Bodies (PLBS)

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ABSTRACT

The Orchidaceae is known as one of the most species-rich families of the plant kingdom. Orchids are generally declining in their natural habitat due to habitat loss and overharvesting for ornamental and medicinal purposes. *Dendrobium* Sabin Blue is widely grown as a cut flower and pot plant and is also popular for its deep violet-blue flowers. Developing these new orchid hybrids is tedious work; it is important to preserve them. Tissue culture and cryopreservation of plants can be employed to produce plantlets on an industrial scale. However, it may lead to genetic instability due to somaclonal variation. This study is to assess the genetic stability of regenerated cryopreserved and non-cryopreserved PLBs in comparison to stock culture PLBs using directed amplification of minisatellite DNA regions (DAMD) and inter-simple sequence repeat (ISSR) DNA molecular analyses. In general, regenerated explants should be identical to the mother plant. Seventeen (17) DAMD primers and twenty (20) ISSR primers were used to assess genetic stability between the 4-week-old cryopreserved/non-cryopreserved PLBs and the PLBs of the parent culture. Finally, DAMD and ISSR analyses confirmed the occurrence of 7% polymorphism and monomorphism, respectively, in the regenerated cryopreserved PLBs. Future studies should include further evaluation of somaclonal variations for long-term maintenance of cryopreserved PLBs.

Key words: *Dendrobium* Sabin Blue, directed amplification of minisatellite DNA regions (DAMD), inter simple sequence repeat (ISSR), polymorphism

INTRODUCTION

Orchids, which belong to the Orchidaceae family, have the greatest commercial and ornamental value. Therefore, storing orchid explants in cryogenics could enable mass breeding for commercial purposes and serve as an important tool in plant breeding (Vettorazzi *et al*., 2019). Orchidaceae, the largest family of flowering plants, is estimated to include 880 genera and over 25,000 species. Orchids are popular and in demand worldwide. Many orchids have high medicinal, ornamental, and cultural value (Popova *et al*., 2016). Besides, several orchids are often used as pot plants. *Dendrobium* is one of the largest genera of Orchidaceae with more than 1500 species and is widely distributed around the world (Zhao *et al*., 2019). Indeed, *Dendrobium* orchids are widely used as cut flowers in the international flower trade (Kuehnle 2007; Sawettalake *et al*., 2017; Zhao *et al*., 2019). The main unique characteristics of *Dendrobium* compared to other pot orchids are their flowering inflorescences, a wide variety of colours, sizes, and shapes, year-round availability, and long flowering periods that can last up to weeks and months (Kuehnle 2007; Sawettalake *et al*., 2017; Zhao *et al*., 2019).

Dendrobium Sabin Blue is a perennial and sympodial hybrid orchid whose parents are *Dendrobium* Blue Angel x *Dendrobium* Sanan Blue. The flowers are round and 7 cm in diameter with deep violet-blue petals and sepals. The *Dendrobium* Sabin Blue hybrid is often grown as a cut flower and pot plant because of its attractive flower colour. *Dendrobium* Sabin Blue hybrids with their dark blue-purple flower colour can contain different pigments. The purple and blue colouration of *Dendrobium* flowers could be due to the anthocyanin pigment, which is a water-soluble flavonoid pigment found in the vacuoles of plant cells (Li *et al*., 2017). The unique flower colour of *Dendrobium* hybrids is one of the most important features that captured the consumers' attention (Li *et al*., 2017). Moreover, the number of orchid hybrids with blue-purple flower colour is limited among breeders, as breeding takes a lot of time (Kuehnle *et al*., 1997). Therefore, it is important to maintain this aesthetic hybrid, *Dendrobium* Sabin

Article History
Accepted: 10 July 2025
First version online: 30 September 2025
Cite This Article:
James Antony, J.J., Zakaria, S., Mubarakh, S.A., Zakaria, R., Embu, E.A. & Subramaniam, S. 2025. DAMD and ISSR DNA molecular analyses of regenerated cryopreserved Dendrobium Sabin Blue's protocorm-like bodies (PLBS). Malaysian Applied Biology, 54(3): 1-14. https://doi.org/10.55230/mabjournal.v54i3.3004

Blue, as the varieties are only common among breeders. Therefore, a plant vitrification solution 2 (PVS2) cryopreservation method was successfully developed in previous studies to conserve this orchid.

Cryopreservation is one of the most efficient techniques for long-term conservation of plant germplasm, where a cryogenic liquid is used to store the plant biological material, and when combined with *in vitro* methodologies, is considered a notable method for safe and long-term germplasm storage by limiting its metabolic activities (Bansal *et al*., 2023). Plant cryobiology has largely developed the technologies using various explants obtained *in vitro*, leading to successful results (Ai *et al*., 2012; Zhang *et al*., 2020; Nausch & Buyel, 2021). Vitrification-based cryopreservation techniques often involve several essential steps, such as preculture, loading, and exposure to PVS2. In turn, these steps could cause abiotic stress and chemical toxicity to the cells, leading to genetic alterations in the cryogenic plants (Ai *et al*., 2012; Galdiano *et al*., 2013; Ibáñez *et al*., 2019; Zhang *et al*., 2020; Nausch & Buyel, 2021). The formation of reactive oxygen species during the cryopreservation steps in turn leads to lipid peroxidation, protein denaturation, and DNA mutations in the cryopreserved plant materials (Galdiano *et al*., 2013; Nausch & Buyel, 2021). As described by Ibáñez *et al*. (2019), ROS-induced DNA damage was found in some plant cultures. Somaclonal variations in regenerated cryopreserved materials include changes in plant morphology, DNA sequences, chromosome number, gene expression, and protein profiles (Kaity *et al*., 2008; Nausch & Buyel, 2021). In general, the genetic stability of plants derived from cryopreservation is assessed using numerous methods such as morphological, cytological, biochemical, and molecular analyses (Srivastava *et al*., 2009; Baranek *et al*., 2010; Konieczny *et al*., 2010; Mallon *et al*., 2010; Ai *et al*., 2012; Zhang *et al*., 2020). Therefore, genetic analyses need to be performed on the regenerated cryopreserved plants to confirm their genetic stability (Galdiano *et al*., 2013; Zhang *et al*., 2020).

PCR-based single primer amplification reaction (SPAR) is an effective method for genetic diversity studies in plants. Several studies that applied this method have detected genetic differences among the plant materials analyzed (Ranade *et al*., 2008; Sharma *et al*., 2011; Bhattacharyya *et al*., 2015a; Chin *et al*., 2019; Konar *et al*., 2019; Goswami *et al*., 2020; Oliya *et al*., 2021). The SPAR molecular methods include random amplified polymorphic DNA (RAPD) (Welsh & McClelland 1990; Williams *et al*., 1990), directed amplification of minisatellite DNA regions (DAMD) (Heath *et al*., 1993) and inter simple sequence repeat (ISSR) (Zietkiewicz *et al*., 1994) are preferred as these markers are generally stable, not dependent on the environment, have high performance, are reproducible and have high accuracy in detecting plant variants (Mahar *et al*., 2011a; Chin *et al*., 2019; Konar *et al*., 2019; Goswami *et al*., 2020; Oliya *et al*., 2021). Moreover, these methods are universal and do not require prior knowledge of the DNA sequences of the plant material (Mahar *et al*., 2011a; Chin *et al*., 2019; Konar *et al*., 2019; Goswami *et al*., 2020; Oliya *et al*., 2021). Yet, the methods of SPAR have been used in genetic studies on various plants such as Morus (Bhattacharya *et al*., 2005), Bauhinia (Rana *et al*., 2007), *Jatropha curcas* (Ranade *et al*., 2008), *Murraya paniculata* (Verma *et al*., 2009), Chenopodium (Rana *et al*., 2010), *Sapindus mukorosii* (Mahar *et al*., 2011b), and *Hibiscus sabdariffa* L. (Konar *et al*., 2019). Various Polymerase Chain Reaction (PCR)-based markers within distinct marker systems have demonstrated effectiveness in studying genetic variability among plant species collections, such as the common bean. Several molecular markers, including RAPD, AFLP, ISSR, SSR, and SCoT, have been effectively utilized in analyzing molecular variability within the common bean (Hromadová *et al*., 2023).

Although RAPD genetic analyses are widely used in plants, one of the main limitations is poor reproducibility, which in turn has been addressed by the ISSR and DAMD markers (Heath *et al*., 1993; Zietkiewicz *et al*., 1994; Sharma *et al*., 2011). Moreover, these two genetic markers (ISSR & DAMD markers) have been widely used to assess genetic fidelity in micropropagated plants such as *Nepenthes khasiana* (Devi *et al*., 2014), *Withania somnifera* (Fatima *et al*., 2015), *Henckelia incana* (Prameela *et al*., 2015), and micropropagated *Dendrobium* Sabin Blue PLBs (Chin *et al*., 2019). Moreover, a comparison between ISSR, DAMD, and RAPD markers in the evaluation of genetic diversity of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) cultivars shows that DAMD and ISSR have a higher success marker rate compared to RAPD (Saidi *et al*. 2023).

Therefore, in this study, ISSR and DAMD molecular markers are used to assess the genetic stability of cryopreserved PLB. Currently, there is no report on the application of ISSR and DAMD markers to PLBs of *Dendrobium* Sabin Blue after cryopreservation.

MATERIALS AND METHODS

Plant material

In vitro cultures of *Dendrobium* Sabin Blue PLBs were obtained from the PVS2 (Plant Vitrification Solution) cryopreservation protocol previously established by our group at Plant Biotechnology Laboratory 310, School of Biological Sciences, USM, Penang. This plant material was used as an explant to initiate the propagation of PLBs for the subsequent experiments. Plant cultures were propagated and maintained on Murashige and Skoog (1962) semi-solid medium supplemented with 1 mg/L BAP (BAP; DUCHEFA, The Netherlands), 20 g/L sucrose, and solidified with 2.75 g/L Gelrite TM (DUCHEFA, The Netherlands). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. PLBs were subcultured every 4 weeks and incubated at 25±2°C under a 16 hr photoperiod (Philips TLD, 36 W, 150μmoL.m^{-2}. s^{-1}).

DNA extraction

Genomic DNA of PLBs was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. Plant DNA was extracted from 3 types of samples, namely regenerated cryopreserved (+ LN), non-cryopreserved (- LN), and PLB stock cultures (S) taken from 4-week-old cultures. The quality and concentration of the extracted DNA were quantified using the Thermo Scientific Nano-Drop™ 1000 spectrophotometer (ASP -2680, ACTGene Inc., New Jersey, USA). The extracted DNA was stored at 4°C before use.

DNA analysis

All three types of DNA samples were screened using 24 DAMD primers based on the references of Devi *et al*. (2014) and Bhattacharyya *et al*. (2015a). However, only 17 primers showed reproducible results, so these primers were used in the subsequent DAMD analysis after primer screening (Table 1). The 20 µL reaction mixture consisted of 30 ng DNA, 0.2 mM deoxyribonucleotide triphosphate (dNTP) mixture, 1× PCR buffer, 1.5 mM magnesium chloride ($MgCl_2$), 1-unit *Taq* DNA polymerase (Econotaq®, separate MgCl 2), 0.5 µM primer, and autoclaved deionised water. DNA amplification was performed in the PCR instrument [T100™ Thermal Cycler (Bio Rad Laboratories, Inc., USA)], with the programme set for initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 92°C for 1 min, annealing at Tm-5°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The reaction profile for the PCR setup was based on Bhattacharyya *et al*. (2015a).

Table 1. List of primers selected for DAMD analysis

Primer	Sequence (5'-3')	GC content (%)	Tm (°C)
URP9F	ATGTGTGCGATCAGTTGCTG	50.0	56.0
URP4R	AGGACTCGATAACAGGCTCC	55.0	56.1
HBV3	GGTGAAGCACAGGTG	60.0	50.0
6_2H (-)	CCCTCCTCCTCCTTC	66.7	50.4
6_2H (+)	AGGAGGAGGGGAAGG	66.7	52.4
M13	GAGGGTGGCGGCTCT	73.3	57.9
URP2R	CCCAGCAACTGATCGCACAC	60.0	59.1
33.6	GGAGGTGGGCA	72.7	44.9
INS	ACAGGGGTGGGG	75.0	49.4
HBV5	GGTGTAGAGAGGGGT	60.0	49.0
HVR	CCTCCTCCCTCCT	69.2	47.6
URP38F	AAGAGGCATTCTACCACCAC	50.0	54.5
URP13R	TACATCGCAAGTGACACAGG	50.0	54.9
URP9F	ATGTGTGCGATCAGTTGCTG	50.0	56.0
URPIF	ATCCAAGGTCCGAGACAACC	55.0	56.8
URP17R	AATGTGGGCAAGCTGGTGGT	55.0	60.1
URP6R	GGCAAGCTGGTGGGAGGTAC	65.0	60.6

Note* The primers' melting temperatures were adapted from the manufacturer, First Base Laboratory Sdn. Bhd (Malaysia)

ISSR analysis

All 3 samples were screened with 30 ISSR primers based on Wang *et al*. (2009) and Bhattacharyya *et al*. (2015a). A total of 20 primers showed clear and reproducible bands, so these primers were used for subsequent ISSR analysis after primer screening (Table 2). The PCR setup followed the protocol outlined by Bhattacharyya *et al*. (2015a) with a reaction volume of 20 µL. The molecular reagent composition was the same as described in the DNA analysis section, except the primer concentration was adjusted to 1 µM. The PCR profile was performed with an initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 92°C for 30 sec, annealing at Tm-5°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 7 min.

Table 2. List of primers selected for ISSR analysis

Primer	Sequence (5'-3')	GC content (%)	Tm (°C)
UBC855	ACACACACACACACACYT	47.2	53.1
UBC827	ACACACACACACACACG	52.9	53.0
125	ACACACACACACACACCA	50.0	54.5
144	ACACACACACACACACGA	50.0	54.5
UBC864	ATGATGATGATGATGATG	33.3	43.6
UBC868	GAAGAAGAAGAAGAAGAA	33.3	43.2
174	ACTGACTGACTGACTG	50.0	47.4
UBC840	GAGAGAGAGAGAGAGAYT	47.2	47.4
134	AGAGAGAGAGAGAGAGAA	44.4	47.5
I4	ACACACACACACACACAG	50.0	52.9
I2	ACACACACACACACACAT	44.4	52.0
UBC825	ACACACACACACACACT	47.1	51.4
UBC818	CACACACACACACACAG	52.9	51.0
UBC834	AGAGAGAGAGAGAGAGYT	47.2	49.2
UBC835	AGAGAGAGAGAGAGAGYC	52.8	50.2
UBC811	GAGAGAGAGAGAGAGAC	52.9	46.8
R	CACACACACACAGT	50.0	44.7
B	GAGAGAGAGAGAGAGAT	47.1	45.4
N	CACACACACACAGG	57.1	46.2
W	ACACACACACACACACAA	44.4	52.2

Note* The primers' melting temperatures were adapted from the manufacturer, First Base Laboratory Sdn. Bhd (Malaysia)

Evaluation of genetic stability

All PCR amplification products were separated on a 1.5% (w/v) agarose gel in 1 × Tris-borate-EDTA (TBE) buffer, with the addition of Redsafe nucleic acid staining solution (iNtRON Biotechnology, South Korea). The PCR products from the cryopreserved and non-cryopreserved PLBs were then scored by counting the similarity index (SI) of the treated PLBs compared to the PLB stock culture. Clear and reproducible bands were defined as 0 for the absence and 1 for the presence of bands. The similarity index (SI) between treatments was calculated using the following formula (Nei & Li, 1979; Asnita & Norzulaani, 2006).

$$SI = \frac{2Nxy}{Nx+Ny}$$

Whereby, SI= Similarity Index;
Nxy Number of monomorphic bands between the stock culture PLBs and regenerated cryopreserved or non-cryopreserved PLBs
Nx = Total number of bands in the PLBs stock culture
Ny = Total number of bands in the regenerated cryopreserved or non-cryopreserved PLBs
SI = 1 indicates monomorphism; SI = 0 indicates polymorphism; SI = 0.1 - 0.9 indicates partial polymorphism.

The polymorphism percentage in regenerated cryopreserved PLBs and regenerated non-cryopreserved PLBs was calculated based on a formula by Blair *et al*. (1999).

$$\text{Polymorphism percentage (\%)} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

RESULTS

DAMD analysis

In the present study, the molecular marker DAMD was used to determine genetic stability in the cryopreserved and non-cryopreserved PLBs. A total of 17 DAMD primers yielded 98, 101, and 105 bands from the stock culture PLBs, regenerated cryopreserved PLBs, and regenerated non-cryopreserved PLBs, respectively, with a range of 200 – 5000 bp. The number of bands per primer ranged from 0 to 9 bands per primer (Tables 3 & 4).

Genetic evaluation between the cryopreserved PLBs and the stock culture PLBs revealed 101 bands in the cryopreserved PLBs [94 monomorphic & 7 polymorphic bands]. In cryopreserved PLBs, the overall percentage of polymorphism was 7.0% (Table 3). Three primers indicated partial polymorphism based on SI index scores in cryopreserved PLBs, namely URP4R, M13, and 6_2H (+). Primer URP4R was recorded with a SI index of 0.8 and a percentage polymorphism of 37.5 %, primer 6_2H (+) with a SI index of 0.4 and a percentage polymorphism of 75.0 % and M13 with a SI index of 0.9 and a percentage polymorphism of 12.5% (Table 3).

The analysis found that 12 primers had an SI (similarity index) of 1, indicating complete similarity in the non-cryopreserved PLBs. Five other primers showed partial polymorphism, meaning there were some genetic differences. These primers were URP4R, 6_2H (+), M13, URP2R, and URP9F. For example, primer URP4R had an SI index of 0.8 and a 37.5% polymorphism rate, while primer M13 had an SI index of 0.9 but showed no polymorphism. Both primers M13 and URP9F produced multiple bands in both stock culture and non-cryopreserved PLBs but exhibited no polymorphic bands, indicating high similarity in the genetic material (Table 4).

ISSR analysis

Genetic analysis using 20 ISSR primers revealed 134 bands in the stock PLBs, 125 bands in the regenerated cryopreserved PLBs, and 128 bands in the regenerated non-cryopreserved PLBs. The size of the PCR-amplified products ranged from 200 to 2000 bp with 2 to 11 bands per primer (Tables 5, 6).

A total of 125 monomorphic bands were produced in both cryopreserved and strain PLBs. Thus, the cryopreserved PLBs showed no polymorphism (0%) and an overall percentage of 100% monomorphism (Table 5). A total of 18 primers gave an SI index of 1, and 2 primers showed partial polymorphism, namely primers B and N. Nevertheless, primers B and N gave an SI index of 0.6, with no polymorphism detected in the cryopreserved PLBs (Table 5).

In contrast, the comparison between non-cryopreserved PLBs and stock culture PLBs revealed 125 monomorphic bands. Additionally, 3 polymorphic bands were observed in the non-cryopreserved PLBs, resulting in an overall polymorphism percentage of 2.0% (Table 6). The ISSR profiles identified 17 primers with an SI index of 1 and 3 primers showing partial polymorphism, specifically primers UBC864, R, and N. Primers UBC864 and N had SI indices of 0.7, with polymorphism rates of 40.0% and 14.3%, respectively. In contrast, primer R had an SI index of 0.8 with no polymorphism (0%) (Table 6). Figure 2 illustrates the banding patterns of cryopreserved and non-cryopreserved PLBs compared to stock culture PLBs using the 6 selected ISSR primers.

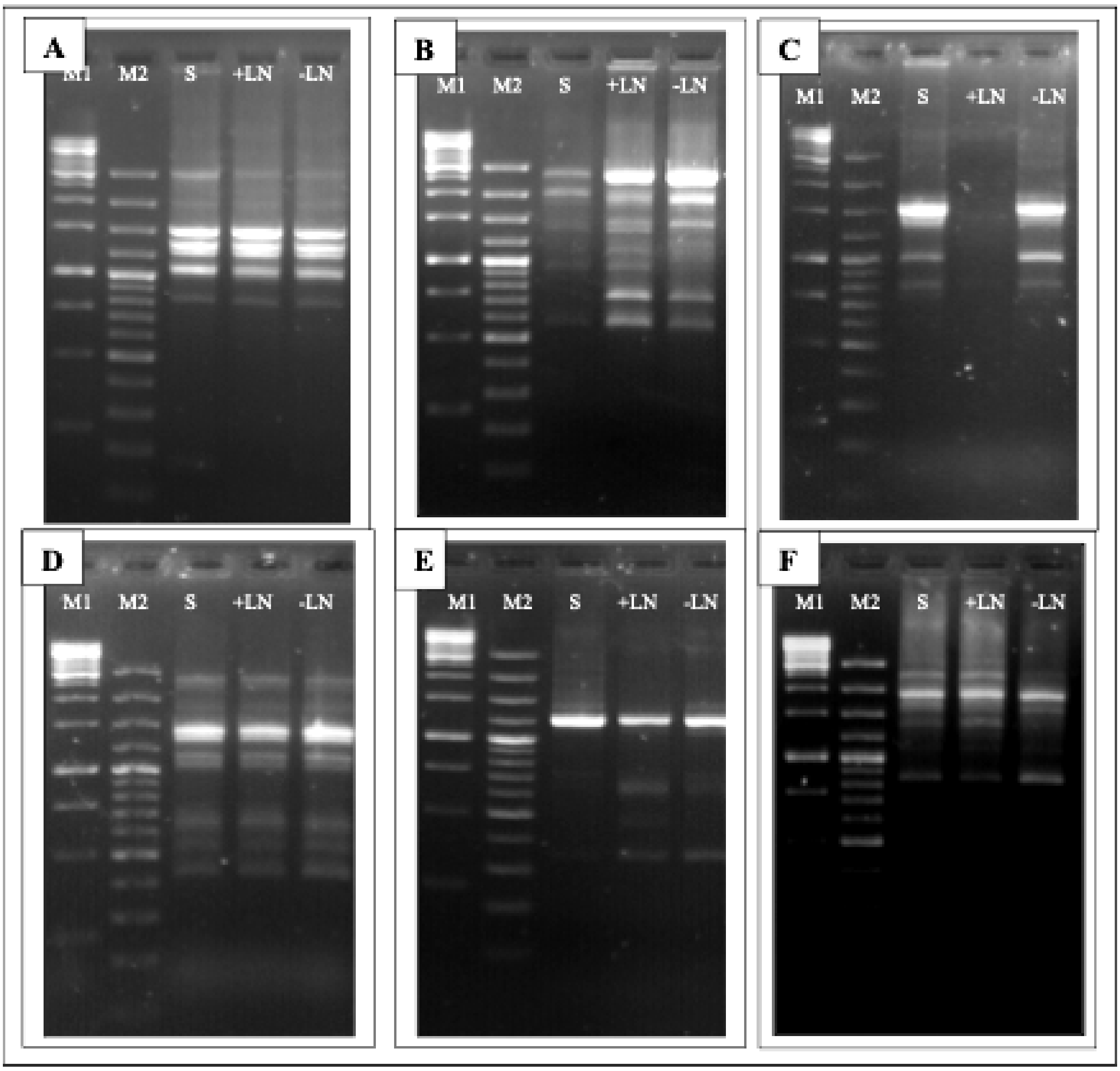

Fig. 1. Banding profile of regenerated cryopreserved PLBs (+LN) and non- cryopreserved PLBs (-LN) compared to stock culture PLBs (S) using DAMD primers (A) URP9F; (B) URP4R; (C) HBV3; (D) 6_2H (-); (E) 6_2H (+); (F) M13 M1; 1 kb ladder, M2; 100 bp plus ladder

Table 3. DAMD analysis of cryopreserved PLBs compared with the stock culture PLBs

Primer	Total number of bands in stock culture PLBs	Total number of bands in cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments (bp)	SI Index	Polymorphism percentage (%)
URP9F	7	7	7	0	800-3000	1	0
URP4R	5	8	5	3	600-3000	0.8	37.5
HBV3	4	0	0	0	800-1500	0	0
6_2H (-)	9	9	9	0	300-3000	1	0
6_2H (+)	1	4	1	3	300-1200	0.4	75
M13	7	8	7	1	800-3000	0.9	12.5
URP2R	4	4	4	0	300-2000	1	0
33.6	6	6	6	0	400-1500	1	0
INS	7	7	7	0	200-1500	1	0
HBV5	6	6	6	0	800-1500	1	0
HVR	4	4	4	0	800-2000	1	0
URP38F	7	7	7	0	700-3000	1	0
URP13R	4	4	4	0	900-3000	1	0
URP9F	8	8	8	0	600-5000	1	0
URPIF Primer URPIF	7	7	7	0	500-3000	1	0
URP17R	8	8	8	0	200-2000	1	0
URP6R	4	4	4	0	300-3000	1	0
Total number of bands	98	101	94	7	-	-	7

Table 4. DAMD analysis of non-cryopreserved PLBs compared with the stock culture PLBs

Primer	Total number of bands in stock culture PLBs	Total number of bands in non-cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments (bp)	SI Index	Polymorphism percentage (%)
URP9F	7	7	7	0	800-3000	1	0
URP4R	5	8	5	3	600-3000	0.8	37.5
HBV3	4	4	4	0	800-1500	1	0
6_2H (-)	9	9	9	0	300-3000	1	0
6_2H (+)	1	6	1	5	300-1200	0.3	83.3
M13	7	6	6	0	800-3000	0.9	0
URP2R	4	5	4	1	300-2000	0.9	20
33.6	6	6	6	0	400-1500	1	0
INS	7	7	7	0	200-1500	1	0
HBV5	6	6	6	0	800-1500	1	0
HVR	4	4	4	0	800-2000	1	0
URP38F	7	7	7	0	700-3000	1	0
URP13R	4	4	4	0	900-3000	1	0
URP9F	8	7	7	0	600-5000	0.9	0
URPIF Primer URPIF	7	7	7	0	500-3000	1	0
URP17R	8	8	8	0	200-3000	1	0
URP6R	4	4	4	0	300-3000	1	0
Total number of bands	98	105	96	9	-	-	9

Table 5. Comparative ISSR analysis between cryopreserved PLBs and the stock culture PLBs

Primer	Total number of bands in the PLBs stock culture	Total number of bands in cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments (bp)	SI Index	Polymorphism percentage (%)
UBC855	6	6	6	0	200-1500	1	0
UBC827	6	6	6	0	500-1500	1	0
125	7	7	7	0	400-1500	1	0
144	9	9	9	0	300-2000	1	0
UBC864	4	4	4	0	800-1500	1	0
UBC868	8	8	8	0	400-2000	1	0
174	6	6	6	0	500-2000	1	0
UBC840	7	7	7	0	300-1200	1	0
134	5	5	5	0	400-1200	1	0
I4	6	6	6	0	700-2000	1	0
I2	8	8	8	0	500-1200	1	0
UBC825	6	6	6	0	200-600	1	0
UBC818	7	7	7	0	500-2000	1	0
UBC834	5	5	5	0	300-900	1	0
UBC835	7	7	7	0	200-1200	1	0
UBC811	4	4	4	0	600-1200	1	0
R	9	9	9	0	300-2000	1	0
B	5	2	2	0	400-1500	0.6	0
N	11	5	5	0	200-2000	0.6	0
W	8	8	8	0	300-1200	1	0
Total number of bands	134	125	125	0	-	-	0

Table 6. Comparative ISSR analysis of non-cryopreserved PLBs with the stock culture PLBs

Primer	Total number of bands in the PLBs stock culture	Total number of bands in non-cryopreserved PLBs	Total number of monomorphic bands	Total number of polymorphic bands	Length of amplified DNA fragments (bp)	SI Index	Polymorphism percentage (%)
UBC855	6	6	6	0	200-1500	1	0
UBC827	6	6	6	0	500-1500	1	0
125	7	7	7	0	400-1500	1	0
144	9	9	9	0	300-2000	1	0
UBC864	4	5	3	2	600-1500	0.7	40
UBC868	8	8	8	0	400-2000	1	0
174	6	6	6	0	500-2000	1	0
UBC840	7	7	7	0	300-1200	1	0
134	5	5	5	0	400-1200	1	0
I4	6	6	6	0	700-2000	1	0
I2	8	8	8	0	500-1200	1	0
UBC825	6	6	6	0	200-600	1	0
UBC818	7	7	7	0	500-2000	1	0
UBC834	5	5	5	0	300-900	1	0
UBC835	7	7	7	0	200-1200	1	0
UBC811	4	4	4	0	600-1200	1	0
R	9	6	6	0	300-2000	0.8	0
B	5	5	5	0	400-1500	1	0
N	11	7	6	1	200-2000	0.7	14.3
W	8	8	8	0	300-1200	1	0
Total number of bands	134	128	125	3	-	-	2

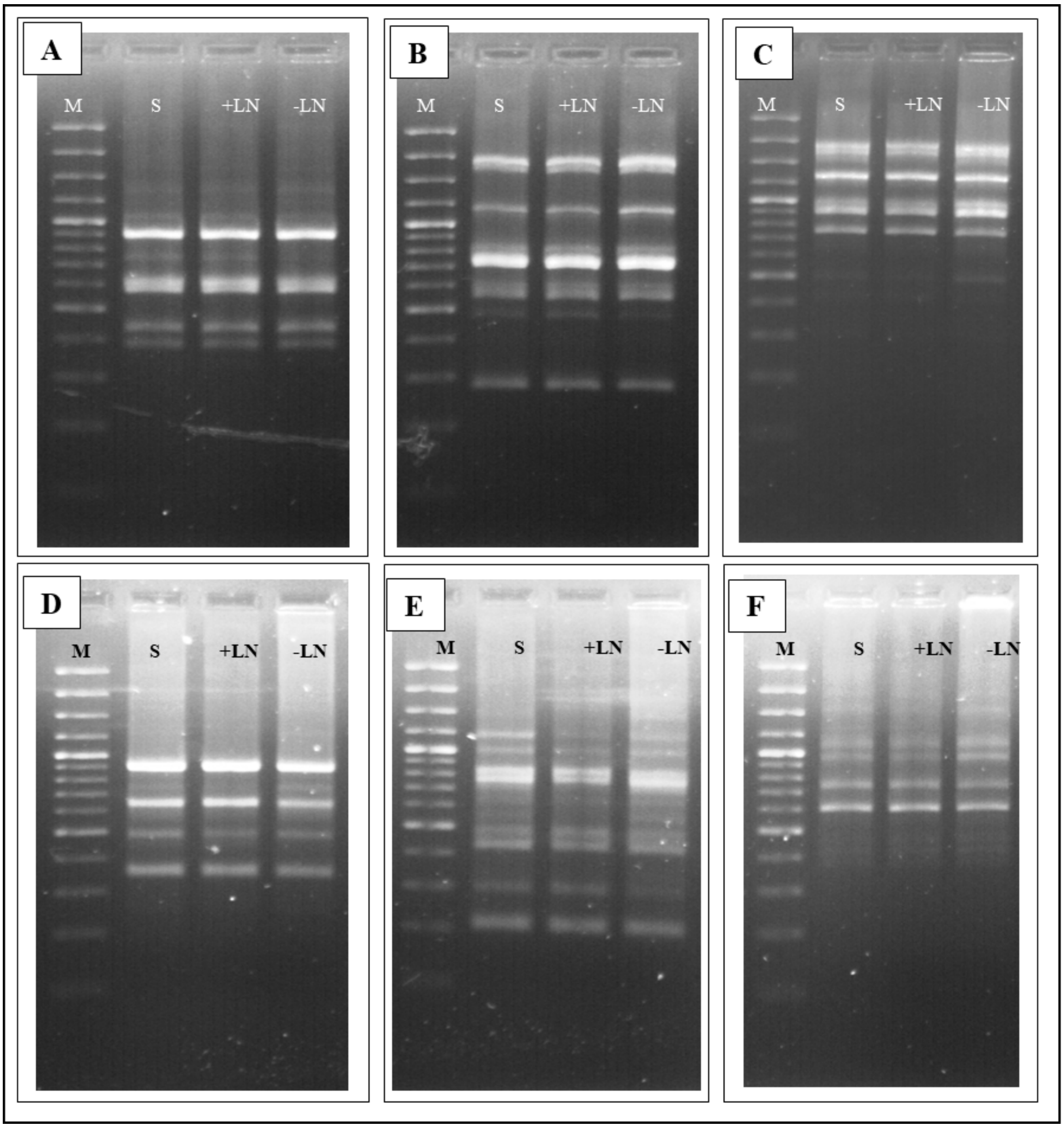

Fig. 2. Banding profile of regenerated cryopreserved PLBs (+LN) and non-cryopreserved PLBs (-LN) compared to stock culture PLBs (S) using ISSR primers (A); 125 (B), 144. (C) UBC818; (D) UBC834; (E) UBC835; (F) UBC811 M; 100 bp plus ladder.

DISCUSSION

PVS2 cryopreservation techniques for vitrification often include steps such as pre-culture, loading, and PVS2 treatment. These steps trigger abiotic stress and chemical toxicity to the cells, resulting in genetic and/or epigenetic changes in cryogenic plants (Zhang *et al*., 2020). In this study, the regenerated cryopreserved PLBs of *Dendrobium* Sabin Blue yielded 93 and 100% monomorphism via DAMD and ISSR analyses, respectively. In addition, 7% polymorphism was detected in the regenerated cryopreserved PLBs via DAMD analysis, and no polymorphism was detected via ISSR analysis. The higher polymorphism in non-cryopreserved PLBs compared to stock culture PLBs may be due to ongoing cellular activity and environmental exposure, which can induce genetic variation, whereas cryopreserved PLBs are subjected to freezing conditions that tend to preserve genetic integrity and minimize changes. According to Cheng *et al*. (2020), the genetic stability was observed in plants post-cryopreservation. For instance, in experiments involving the cryopreservation of protocorm-like bodies (PLBs), research suggests that differences in polymorphism between non-cryopreserved and stock culture PLBs could arise due to the higher level of cellular activity and environmental exposure in non-cryopreserved PLBs. This exposure potentially induces genetic variation, as noted in protocols using encapsulation-dehydration methods. By contrast, cryopreservation subjects PLBs to freezing, which tends to preserve genetic integrity and reduce genetic changes. A similar observation was made during the cryopreservation of *Dendrobium* species, where post-cryopreservation analyses showed minimal genetic differences between cryopreserved and non-cryopreserved samples using ISSR and RAPD markers (Cheng *et al*., 2020). A similar result was obtained with a percentage of 5.78% polymorphism in cryopreserved chrysanthemum tips by RAPD analysis (Martin *et al*., 2011). The genetic stability was tested by using 40 ISSR primers, with somaclonal variants as a possible limitation to plant *in vitro* multiplication. The PCR amplification profiles obtained from all the investigated propagules (calli, meristemic clumps & regenerated plantlets) were equivalent to those obtained from the mother plants, indicating that the developed individuals using regeneration methods presented here were homogenous (Quijada-Rivera *et al*., 2023*)*.

Instead, plants regenerated from cryopreserved shoot tips of *Malus* × *domestica* did not show polymorphic bands in ISSR analysis (Liu *et al*., 2008). Recent studies have confirmed that plants regenerated from cryopreserved shoot tips, such as those of *Malus* × *domestica* (apple), maintain high genetic stability. Specifically, the cryopreservation process does not introduce polymorphic bands when analyzed using ISSR markers, confirming genetic fidelity. This aligns with broader findings in the cryopreservation of other plant species like *Populus tremula* × *Populus tremuloides*, where methods ensured the preservation of genetic integrity post-cryopreservation. Such consistency in results supports the reliability of cryopreservation for maintaining true-to-type plant regeneration without genetic variation (Zhang *et al*., 2015; Wang *et al*., 2018). In addition, regenerated cryopreserved plants of *Humulus lupulus* showed no genetic variation compared to the mother plant in AFLP analysis (Peredo *et al*., 2008). According to Sharma *et al*. 2022, genetic stability was also maintained in regenerated plants from cryopreserved shoot tips, such as *Paeonia lactiflora* (Seo *et al*., 2007), *Dioscorea rotundata* (Mandal *et al*., 2008), *Hypericum perforatum* (Skyba *et al*., 2010), and *Carica papaya* (Kaity *et al*., 2013). Furthermore, no polymorphic bands were detected in the cryogenic kiwi fruit samples of the cultivar ‘Yuxiang’ (*Actinidia chinensis* var. deliciosa) after the droplet vitrification method using ISSR analyses (Zhang *et al*., 2020). In fact, cryopreserved strawberries (*Fragaria* x *ananassa* Duch.) of six accessions were considered genetically stable by molecular assessment using ISSR markers in research done by Bae *et al*. (2022).

Genetic analyses performed on seedlings regenerated from cryopreserved *Dendrobium candidum* PLBs (Yin & Hong, 2009) and cryopreserved seeds of *Dendrobium* hybrid ‘Dong Yai’ (Galdiano *et al*., 2013) showed similarity with the respective control plants. Furthermore, Ding *et al*. (2008) demonstrated that conserved *Dendrobium officinale* plantlets had 96 polymorphic bands out of a total of 109 bands using sequence-related amplified polymorphism (SRAP) (Ding *et al*., 2008). However, Popova *et al*. (2016) noted that few studies have been conducted on genetic stability after cryopreservation of orchids using protocorms and PLBs as explants. Recent studies on the ploidy level of *Vanda coerulea* plants regenerated from cryopreserved protocorms have shown that the encapsulation-dehydration method does not affect the ploidy stability. Flow cytometric analyses revealed that the regenerated plantlets maintained the same ploidy levels as the non-cryopreserved counterparts, ensuring genetic stability throughout the process. This method has been demonstrated to be effective in preserving the genetic fidelity of various orchid species, including *Vanda coerulea*, by maintaining stable morphological characteristics and growth patterns post-cryopreservation (Jitsopakul *et al*., 2008; Thammasiri *et al*., 2022). Furthermore, Antony *et al*. (2012) observed that regenerated PLBs of *Dendrobium* Bobby Messina exhibited genetic stability after a PVS2-based vitrification method using RAPD analysis (Antony *et al*., 2012). Similarly, RAPD analysis confirmed that genetic stability was maintained in regenerated protocultures of *Dendrobium virgineum* (Maneerattanarungroj 2009) and *Vanda coerulea* PLBs (Jitsopakul *et al*., 2011) after cryopreservation by encapsulation-dehydration and droplet vitrification, respectively. In addition, plants from one year of *in vitro* growth, both cryopreserved and non-cryopreserved samples, revealed no change in ploidy level in Brazilian orchids such as *Cattleya harrisoniana* × *Cattleya walkeriana* hybrid and *Cattleya tigrine* by the flow cytometry method (Vettorazzi *et al*., 2019). Similarly, RAPD and SCoT molecular analyses confirmed the genetic stability of regenerated cryopreserved PLBs Aranda Broga Blue Orchid following the droplet vitrification method, with no observed polymorphism compared to control PLBs (Khor *et al*., 2020). Furthermore, RAPD analyses have shown that genetic stability was maintained in micropropagated plants of *Rhynchostylis* retusa (L.) compared to their mother plant (Oliya *et al*., 2021).

However, in this study, different results were obtained, with DAMD analysis showing polymorphism in the regenerated cryopreserved PLBs, while ISSR analysis showed no polymorphism. The use of additional markers is often used to amplify different sections of the genome (Chin *et al*., 2019). This enables a more comprehensive investigation of somaclonal variation compared to the use of a single molecular analysis (Bhattacharya *et al*., 2005; Martín *et al*., 2011; Fatima *et al*., 2015). The PCR-based single primer amplification reaction method (SPAR), which includes RAPD, ISSR, and DAMD analyses, has gained prominence and is widely used for analyses of genetic diversity and genetic stability of micropropagated and cryopreserved seedlings due to its speed and reliability (Fatima *et al*., 2015). In fact, DAMD and ISSR markers are said to be very useful tools for hereditary diversity studies in plants as they exhibit a comprehensive depiction of the degree of diversity (Rajan *et al*. 2022).

A similar result was observed in RAPD analysis of regenerated cryopreserved shoot tips of chrysanthemum, which indicated a percentage of 5.8% polymorphism, while AFLP analysis showed a percentage of 40.1% genetic variation (Martín *et al*., 2011). Furthermore, the absence of variation in the genetic composition of the regenerated cryopreserved somatic embryo of *Quercus suber* was confirmed by SSR analysis, while AFLP analysis revealed polymorphism (Fernandes *et al*., 2008). Bhattacharyya *et al*. (2015b) highlighted that the molecular method of Start Codon Targeted Polymorphism (SCoT) was able to detect genetic variability in micropropagated plants of *Dendrobium thyrsiflorum* compared to the ISSR-based method (Bhattacharyya *et al*., 2015b). In particular, ISSR markers consistently demonstrated greater genetic diversity compared to RAPD markers (Ding *et al*., 2009). Yang *et al*. (2023) utilized both methods to analyze genetic diversity among 24 different germplasms of *Dendrobium officinale*, highlighting the superiority of ISSR in detecting variability. This finding aligns with previous research, underscoring the importance of ISSR markers in germplasm conservation and breeding programs (Yang *et al*., 2023). On the other hand, methylation-sensitive amplified polymorphism (MSAP) analyses revealed variations based on DNA methylation of *in vitro* excised nodal segments of *Mentha* x *piperita* L. plant samples subjected to an encapsulation-dehydration cryopreservation protocol, although RAPD and AFLP analyses showed complete genetic stability (Ibanez *et al*., 2019). In addition, genetic stability was shown to be maintained in *Actinidia* spp. Plants obtained by the droplet vitrification cryopreservation method using ISSR and AFLP. However, MSAP analyses revealed a DNA methylation of 1.6% in the cryopreserved plants after they were reintroduced under greenhouse conditions (Zhang *et al*., 2020). The findings in other plants are consistent with a recent report on the genetic stability evaluation of *in vitro* developed *Ficus carica* var. Black Jack plantlets grown on woody plant medium containing 20 M BAP + 8 M IAA under various light treatments (normal fluorescent white light & four different LED spectra), which revealed a significant similarity (97.87%) using ISSR markers. The research associated a minor polymorphism with the error dynamics of a PCR procedure (Quijada-Rivera *et al*., 2023).

Since the DAMD method is performed with higher PCR stringencies, the band profile result is more reproducible compared to other molecular-based methods (Bhattacharya & Ranade, 2001; Chin *et al*., 2019). The current study of 27 mulberry cultivars showed the highest percentage of polymorphic bands in the molecular analysis method DAMD compared to other SPAR methods based on SSR, ISSR, and RAPD (Bhattacharya *et al*., 2005; Al-Mamun *et al*., 2023). In micropropagated seedlings of *Withania somnifera* L., the DAMD primers showed a higher percentage of monomorphic results compared to the RAPD method (Fatima *et al*., 2015). Similarly, the DAMD molecular marker was successful in determining genetic stability in *Vitex negundo* (Schmitz & Lorz, 1990), *Aegle marmelos* (Mishra *et al*., 2008), and *Hibiscus sabdariffa* L. (Konar *et al*., 2019). Moreover, the DAMD marker was more competent than the ISSR marker in detecting a higher percentage of polymorphism in micropropagated protocorm-like bodies of *Dendrobium* Sabin blue (Chin *et al*., 2019). In addition, the DAMD marker was more sensitive and precise compared to SCOT and ISSR markers for the detection of polymorphism in the cryopreserved axillary buds of *L. discolor* (Rajan *et al*. 2022). This confirms that the DAMD marker is a well-suited method for determining genetic variation in plants.

The polymorphism (7%) detected in regenerated cryopreserved PLBs in the present study using DAMD analysis might be related to the enhanced somatic embryogenesis in regenerated cryopreserved PLBs. The overall stages of cryopreservation could contribute to the possible outcome of somaclonal variations. These stages include tissue culture preparation of plant material for cryopreservation, the cryopreservation process itself, and subsequent freezing and regeneration after thawing. The genetic modification could therefore originate from the cryopreservation and tissue culture stages (Khor *et al*., 2020). In addition, according to Bae *et al*. (2022), the presence of polymorphic bands could also be potentially caused by transposable elements (TEs) or point mutations.

In addition, cryopreserved *Zingiber officinale* (ginger) confirms that regenerants exhibit no genetic variation when analyzed using ISSR and RAPD markers, even though there are observable differences in shoot regrowth after cryopreservation. This suggests that the cryopreservation process maintains genetic stability despite physical variations in growth, which is crucial for ensuring the consistency of regenerated plants for conservation or commercial purposes. These findings are consistent with studies on other Zingiberaceae species, which emphasize the importance of cryopreservation protocols in preserving genetic integrity (Yamuna *et al*., 2007; Sharma *et al*., 2022; Chakraborty *et al*., 2023). In addition, plants from cryopreserved chrysanthemum (*Chrysanthemum* × *grandiflorum* /Ramat. /Kitam.) yielded shorter plants and smaller leaf size compared to the control, although the RAPD and ISSR analyses revealed a lower level of genetic variability within the cryopreserved plants (Kulus *et al*., 2019). Although PCR-based genetic markers are most commonly used to assess genetic changes following cryopreservation, conclusions drawn from the results on plant genetic stability need to be carefully evaluated. Regardless of the type of molecular markers used, very few parts of plant genomes are examined (Wang *et al*., 2014; Kulus *et al*., 2019). Moreover, the genetic variation detected may be in the non-coding regions and may not affect plant growth and development (Kaity *et al*., 2008).

Nevertheless, the DAMD marker is widely used as a stable marker for genetic variation analysis in plants, which is more accurate compared to other SPAR-related methods such as RAPD and ISSR (Devi *et al*., 2014; Largia *et al*., 2015). Similarly, DAMD analysis revealed a higher percentage of polymorphism compared to RAPD and ISSR analyses, confirming that DAMD is the most suitable molecular marker for determining genetic variation in the regenerated and micropropagated plants of *Nepenthes khasiana* Hook. F (Devi *et al*., 2014). Since the molecular marker DAMD is considered a suitable marker for assessing genetic variability in plants, this explains the results of the current study, where polymorphism was detected only in the regenerated cryopreserved PLBs of *Dendrobium* Sabin Blue via DAMD analysis. However, analyses of genetic variation in sorghum (*Sorghum bicolor* (L.) Moench) genotypes showed that RAPD analyses revealed 94% polymorphism, which outperformed the other two marker methods, ISSR (86.9%) and DAMD (72.8%) (Satish *et al*., 2016). When examining the germplasm collections of *Trichosanthes dioica* Roxb, RAPD analysis proved superior to ISSR and DAMD analyses in determining the percentage of polymorphic loci. The likely reason for this could be the broad genome coverage provided by the RAPD markers, which allows assessment of the entire genetic make-up, whereas the target regions of the ISSR and DAMD markers are only within the repetitive sequences (Adhikari *et al*., 2020). Instead, long-term *in vitro* cultivated germplasm lines of *Bacopa monnieri* (L.) showed uniform profiles of ISSR and DAMD markers, demonstrating the genetic stability of the obtained germplasm lines (Largia *et al*., 2015). These variable responses prove that molecular analyses or different markers used can be genotype-specific in the plants tested.

CONCLUSION

DAMD analysis revealed 7% polymorphism in the regenerated cryopreserved PLBs, while ISSR analysis revealed no polymorphism compared to the control mother plant. The use of ISSR and DAMD markers to evaluate the genetic stability of *Dendrobium* Sabin Blue regenerated cryopreserved PLBs as cumulative markers is, therefore, considered more robust and accurate than the use of individual DNA marker analyses.

ACKNOWLEDGEMENTS

The authors would like to thank the financial support from the Malaysian Ministry of Higher Education for FRGS 2014 Grant (203/PBIOLOGI/6711456) and My Ph.D. Scholarship. Authors also present gratitude to Universiti Sains Malaysia and Universiti Putra Malaysia Sarawak for supporting this study.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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