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Establishment of In Vitro Regeneration Protocol for Sabah's Jewel Orchid, *Macodes limii* J.J. Wood & A.L. Lamb

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Abstract: Habitat disturbance and excessive collection of wild orchids from their natural habitat have threatened many orchids species at risk of extinction. In this study, the in vitro regeneration protocol for *Macodes limii*, a jewel orchid endemic to Sabah was established. The effects of explant source and plant growth regulators (PGRs) including naphthaleneacetic acid, picloram, 2,4-dichlorophenoxyacetic acid, 6-benzylaminopurine, kinetin, and thidiazuron on the in vitro regeneration capacity of *M. limii* plantlets were examined. Both factors showed a significant interaction in promoting axillary shoot formation. Nodal explants from the third and fourth positions cultured with 1.0 mg/L TDZ, induced 95% of shoot regeneration, with an average of three shoots/explant (1.6–1.8 cm of shoot length) after 90 days of culture. The well-developed plantlets went through an acclimatization phase for 60 days with a 60% of survival rate. An inter simple sequence repeat (ISSR) marker analysis confirmed the genetic stability of the in vitro regenerated plants to the mother plant. The successfully acclimatized plantlets were finally transferred to Poring Orchid Conservation Centre for reintroduction. The established protocol provides the means for large-scale production of this endemic jewel orchid, as well as a basis for further research aimed at the conservation and genetic improvement of this plant.

Keywords: Orchidaceae; micropropagation; plant growth regulators; node position; genetic homogeneity; ISSR markers

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1. Introduction

Orchids are commonly known for their beautiful and distinctive types of flowers, and some of them are appreciated for their beautiful foliage or known as "jewel orchids". Jewel orchid is a group of orchids that are highly prized for their unique leaf morphology [1] and it comprises orchids from several genera including *Anoectochilus*, *Dossinia*, *Goodyera*, *Ludisia*, and *Macodes*. Jewel orchids such as *Anoectochilus formosanus*, *A. roxburghii* and *A. koshunensis* have been used in traditional Chinese medicine and known as the "King of Medicine" [2,3]. *Macodes limii* J.J. Wood & A.L. Lamb is a jewel orchid that belongs to the genus *Macodes* Lindl. (Subtribe Goodyerinae, tribe Cranichideae, subfamily Orchidoideae). *Macodes limii* is endemic to Sabah, Borneo, and the distribution is restricted to the area of hill forest, lower montane ridge forest at elevation ranges between 250 m to 1000 m above sea level and restricted to the ultramafic substrate [4].

Conventional propagation of jewel orchid through stem cutting resulted in a low propagation rate and slow growth [5]. Seed propagation through symbiotic and asymbiotic seed germination that have been introduced in several jewel orchids was less desirable due

to the long juvenile period before flowering as reported in *Goodyera schlechtendaliana* [6], *Haemaria discolor* [7], *A. formosanus* [8,9] and *M. limii* [10], besides leading to the production of heterozygous plants [11]. Alternatively, micropropagation, a rapid clonal propagation has been a recommended approach implemented for years in the orchid floriculture industry as well as for conservation [12,13]. The effect of explant types such as shoot tip and the nodal segment on micropropagation of jewel orchids was reported previously in *A. elatus* [14], *A. roxburghii* [15], *A. formosanus* [16] and *Ludisia discolor* [17]. Furthermore, the incorporation of plant growth regulators (PGRs) in a culture medium at suitable concentration and combination was reported to be beneficial in the shoot multiplication of *A. setaceus* [18,19] and *A. elatus* [20].

A genetic fidelity screening is important to observe the genetic variations among micropropagated plants and mother plant for quality control in plant tissue culture. The type of explant, concentration and types of growth regulators, number and duration of subcultures might influence genetic and epigenetic functions in plant tissue and might cause somaclonal-variation-based changes in propagated plants [21]. Molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), start codon targeted (SCoT) and amplified fragment length polymorphism (AFLP) have been used to assess the genetic stability in micropropagated plants of several orchid species [22–25]. Among these, inter simple sequence repeat (ISSR) markers have successfully revealed a high monomorphism percentage of the in vitro propagated plants to be similar to the mother plants in jewel orchids including *A. formosanus* [26,27] and *A. elatus* [28,29]. These findings suggested that genotypically stable true-to-type plants may be utilized in the industrial commercial production as well as germplasm conservation.

Macodes limii has a unique characteristic of ten distinctive sparkling golden-yellow primary nerves on blackish and purple-green background leaves. However, studies on the propagation as well as conservation of this plant were poorly conducted due to its stringent growth requirement, limited availability due to habitat destruction, and indiscriminate collection from its natural habitat for trading purposes. Therefore, the present study aimed to establish a micropropagation protocol by studying the effects of node positions and PGRs treatments on the in vitro regeneration of *M. limii* plantlets, and subsequently measuring the genetic stability among micropropagated plants and mother plant. The micropropagation protocol established from this study can be used to scale up the production as well as to support the conservation of this native species.

2. Materials and Methods

2.1. Establishment of In Vitro Plantlets

Macodes limii (Figure 1A) plants were collected from their natural habitat in the Kota Belud-Ranau area, and the species was identified by Mr. Jamirus Jamian and prior deposited at the Sandakan Herbarium of Forestry Research Centre, Sandakan, Sabah (specimen no: UMSDD-001-005/2018). For surface sterilization, plants were gently brushed and placed under a continuous flow of tap water to remove solid dirt particles. Stem segments were then sterilized in 70% (*v*/*v*) ethanol for 20 s, followed by the treatment of 2% (*v*/*v*) sodium hypochlorite (Clorox®) for 15 min, and finally rinsed three times with sterilized distilled water. The sterilized nodal explants were cultured on half-strength of Murashige and Skoog (MS) media [30], 3% (*w*/*v*) sucrose, and 1% (*w*/*v*) activated charcoal.

2.2. Effects of Node Position and PGRs

To study the effect of node positions on in vitro shoot multiplication, explants were excised from the 10-month-old in vitro grown plantlets to four node positions counted from the tip of the plant; N1 referred to shoot tip and N2, N3, and N4 referred to the 2nd, 3rd, and 4th node, respectively (Figure 1B,C). The procedure was conducted in the morning for all treatments to minimize the regulation of endogenous phytohormones during nodal excision [31]. All explants were transferred to a glass jar containing 50 mL of half-strength MS medium with 1% (*w*/*v*) activated charcoal, 3% (*w*/*v*) sucrose, 0.3% (*w*/*v*) of Gelrite,

and supplemented with various types of PGRs including auxin such as α-naphthalene acetic acid (NAA), picloram, 2,4-dichlorophenoxyacetic acid (2,4-D), and cytokinin such as 6-benzylaminopurine (BAP), kinetin, and thidiazuron (TDZ) at 1.0 mg/L, respectively. Each treatment consisted of one node with five replicates. Medium devoid of growth regulators served as control. The pH of the medium was adjusted to pH 5.7 with 0.1 N of HCl or NaOH before autoclaving at 121 °C, 101.325 kPa for 20 min. All cultures were incubated for 90 days at 25 ± 2 °C under a 12 h photoperiod with cool white fluorescent lamps (150 µmol m^{-2} s^{-1}). Explants were subcultured three times onto fresh media at 4-week intervals.

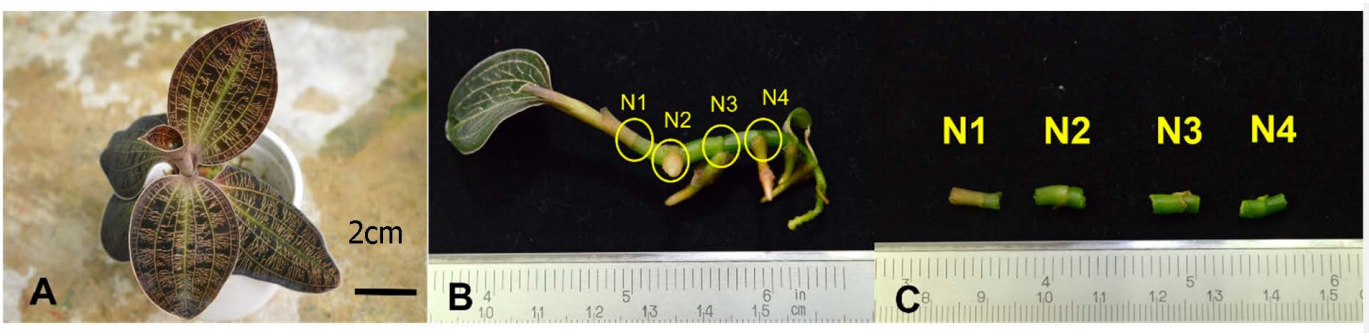

Figure 1. Source of explants for in vitro regeneration of *M. limii.* (**A**) Wild plant of *M. limii;* (**B**) In vitro grown *M. limii* plantlet (starting material); (**C**) Defoliated explants with different node positions.

2.3. Rooting and Acclimatization of Plantlets

To promote root initiation, the in vitro raised shoots of *M. limii* were rooted on half-strength MS medium with 1% (*w*/*v*) activated charcoal, 3% (*w*/*v*) sucrose, 0.3% (*w*/*v*) of Gelrite, and supplemented with indole-3-acetic acid (IAA) at 1.0 mg/L. The cultures were incubated for another 60 days at 25 ± 2 °C under a 12 h photoperiod with cool white fluorescent lamps (150 µmol m^{-2} s^{-1}). Regenerated plantlets of *M. limii* with roots were removed from the glass jars and then washed thoroughly with distilled water to remove the residual medium gel. The plantlets were then transferred to a (5 × 7 cm) plastic pot filled with perlite, charcoal, and sphagnum moss (1:1:1). The plantlets were maintained at 30 °C in a greenhouse nursery with a relative humidity of 70–80% for two months. The successfully acclimatized plantlets were transferred back to the Poring Orchid Conservation Centre (POCC), Ranau for reintroduction.

2.4. Analysis of Genetic Fidelity Using ISSR Primers

Genomic DNA was extracted from the young leaves of the mother plant and eight randomly selected in vitro regenerated hardened plants (10 months old) using a salting-out procedure [32]. For genetic stability assessment, the five ISSRs primers (UBC 808, UBC 835, UBC 836, UBC 841, and UBC 842) [26,27] used in this study were purchased from Integrated DNA Technologies, Singapore. PCR was performed in a total of 15 µL reaction containing 100 ng DNA, 0.48 µM of each primer and 1X Power Taq (Bioteke, Wuxi, China). DNA amplification was performed in a thermocycler programmed for 35 cycles at 30 s (90 °C), 30 s (50 °C), and 50 s (72 °C). The PCR products were resolved on a 2% (*w*/*v*) agarose gel electrophoresis using 1X Tris–Acetate–Ethylenediamine tetraacetic acid buffer.

2.5. Histology and Scanning Electron Microscopy (SEM) of Axillary Shoot Induced from Nodal Explant

Tissues for histological observations were fixed in a solution of formaldehyde alcohol acetic acid (95% (*v*/*v*) ethyl alcohol + glacial acetic acid + formaldehyde + water, 10:1:2:7), dehydrated in a tertiary butyl alcohol series, embedded in paraffin wax, sectioned at 10 µm thickness and stained with 0.5% (*v*/*v*) safranin-O and 0.1% (*v*/*v*) Fast Green. Visualization was carried out using a light microscope (Olympus BX-53, Tokyo, Japan). For SEM, a scan-

ning electron microscope (S-3400N; Hitachi, Tokyo, Japan) was used for the examination and photography of the specimens.

2.6. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistic version 28 (SPSS Inc., Chicago, IL, USA). A factorial two-way analysis of variance (ANOVA) was used to evaluate the effects of two independent factors, namely node position and PGRs treatment, on the in vitro regeneration capacity. Each treatment consisted of five replicates, and the experiment was repeated two times. Data are presented as mean and were compared with Tukey's test at a 5% probability level.

3. Results

3.1. Effects of Node Position and PGRs on In Vitro Regeneration

The effects of node position and PGRs on the in vitro regeneration of *M. limii* were investigated. The highest shoot proliferation response (95%) was observed on N3 and N4 explants cultured on half-strength MS basal medium supplemented with 1.0 mg/L of TDZ (Table 1). The two-way ANOVA showed significant effects on the node position (N), PGRs treatment (P), and the interaction between them (N × P) on the average number of shoot production (Table 2). An average of three new shoots were produced from the N3 or N4 explants cultured on half-strength MS medium supplemented with 1.0 mg/L TDZ after 90 days of culture (DAC). In this study, it was obvious that the N1 explant was less responsive in shoot proliferation compared to the N2, N3, and N4 explants. Two types of regeneration pathways were observed between N1 (shoot tip) and N2, N3, and N4 explants (second, third, and fourth nodes). For N1, the shoot elongation was observed from the excised part and eventually developed into leaf after 90 DAC (Figure 2A,B). Meanwhile, for explants N2–N4, a direct organogenesis through a shoot bud formation was observed. It began with a formation of a white bulge (axillary bud) from the swollen node within 14 DAC, followed by a differentiation into multiple shoots (Figure 2C–G). The histological and SEM observations showed the presence of meristematic cells (axillary shoot) developing from the nodal explant and later forming microshoots through direct organogenesis (Figure 2D–G). The formation of root hair on the nodal explants was also observed from the early stage of shoot multiplication (Figure 2F,G).

The highest shoot length was observed on explant N4 cultured on 1.0 mg/L TDZ with an average of 1.8 ± 0.4 cm shoot length after 90 DAC (Table 1). Besides this treatment, the control medium with no addition of any PGRs also gave a promotive effect on shoot length. The current finding indicates that even though node position influenced the shoot proliferation response and the number of shoots produced, there was no significant difference among them in terms of shoot length (Table 2). Meanwhile, the result also revealed that explants cultured on 1.0 mg/L of NAA gave the least response on shoot proliferation as well as shoot length.

3.2. In Vitro Rooting and Acclimatization of M. limii Plantlets

Microshoots regenerated from all explants (1.4–1.8 cm length) (Figure 2G), were excised carefully and cultured on half-strength MS basal medium supplemented with 1.0 mg/L IAA. After eight weeks of culture, the microshoots were well-developed with two leaves (1.0 cm length) and two to three roots (0.5–1.0 cm length) per responded explant, with a 100% rooting response (Figure 2H). The well-developed plantlets of *M. limii* were selected and acclimatized in a pot containing perlite, charcoal, and sphagnum moss (1:1:1) and maintained under greenhouse conditions for 60 days, achieving a 60% of survivability (Figure 2I). The healthy acclimatized plantlets were selected and transferred back to POCC, Ranau for reintroduction.

Table 1. Effects of PGRs and explant node position on in vitro regeneration of *M. limii* after 90 days of culture.

PGRs	Node Position	% Shoot Formation	No. of Shoots	Shoot Length (cm)
Control	N1	35	$0.7 \pm 0.5^{\text{defgh}}$	$1.6 \pm 1.1^{\text{ab}}$
	N2	75	$1.4 \pm 0.5^{\text{bcdefgh}}$	$1.6 \pm 0.5^{\text{ab}}$
	N3	85	$1.9 \pm 0.7^{\text{abcdefg}}$	$1.4 \pm 0.5^{\text{abc}}$
	N4	60	$2.1 \pm 1.5^{\text{abcde}}$	$1.2 \pm 0.8^{\text{abcde}}$
1.0 mg/L NAA	N1	0	0.0^{h}	0.00^{g}
	N2	35	$0.9 \pm 1.3^{\text{cdefgh}}$	$0.2 \pm 0.3^{\text{fg}}$
	N3	20	$0.4 \pm 0.5^{\text{fgh}}$	$0.3 \pm 0.4^{\text{efg}}$
	N4	15	$0.3 \pm 0.5^{\text{gh}}$	$0.2 \pm 0.3^{\text{fg}}$
1.0 mg/L Picloram	N1	40	$0.8 \pm 0.4^{\text{defgh}}$	$1.3 \pm 0.8^{\text{abcd}}$
	N2	60	$1.2 \pm 0.8^{\text{cdefgh}}$	$0.8 \pm 0.4^{\text{bcdefg}}$
	N3	60	$1.2 \pm 0.8^{\text{cdefgh}}$	$0.8 \pm 0.4^{\text{bcdefg}}$
	N4	50	$1.0 \pm 0.9^{\text{cdefgh}}$	$0.7 \pm 0.6^{\text{bcdefg}}$
1.0 mg/L 2,4-D	N1	20	$0.4 \pm 0.5^{\text{fgh}}$	$0.6 \pm 0.8^{\text{cdefg}}$
	N2	60	$1.2 \pm 1.0^{\text{cdefgh}}$	$0.9 \pm 0.8^{\text{abcdefg}}$
	N3	80	$2.2 \pm 1.2^{\text{abcde}}$	$1.2 \pm 0.5^{\text{abcde}}$
	N4	30	$1.0 \pm 1.6^{\text{cdefgh}}$	$0.4 \pm 0.5^{\text{defg}}$
1.0 mg/L BAP	N1	40	$0.8 \pm 0.4^{\text{defgh}}$	$0.8 \pm 0.7^{\text{bcdefg}}$
	N2	80	$1.8 \pm 0.8^{\text{abcdefg}}$	$0.7 \pm 0.3^{\text{bcdefg}}$
	N3	80	$2.0 \pm 1.1^{\text{abcdef}}$	$0.9 \pm 0.7^{\text{abcdefg}}$
	N4	70	$2.2 \pm 1.4^{\text{abcde}}$	$0.5 \pm 0.3^{\text{cdefg}}$
1.0 mg/L Kinetin	N1	30	$0.6 \pm 0.5^{\text{efgh}}$	$0.7 \pm 0.7^{\text{bcdefg}}$
	N2	80	$1.8 \pm 0.8^{\text{abcdefg}}$	$0.7 \pm 0.4^{\text{bcdefg}}$
	N3	80	$2.5 \pm 1.4^{\text{abc}}$	$0.6 \pm 0.4^{\text{cdefg}}$
	N4	80	$2.2 \pm 1.2^{\text{abcde}}$	$0.7 \pm 0.4^{\text{bcdefg}}$
1.0 mg/L TDZ	N1	40	$0.8 \pm 0.4^{\text{defgh}}$	$1.0 \pm 0.7^{\text{abcdef}}$
	N2	85	$2.3 \pm 1.1^{\text{abcd}}$	$1.4 \pm 0.2^{\text{abc}}$
	N3	95	$3.1 \pm 1.0^{\text{a}}$	$1.6 \pm 0.5^{\text{ab}}$
	N4	95	$3.0 \pm 1.1^{\text{ab}}$	$1.8 \pm 0.4^{\text{a}}$

Data are means from two repeated experiments. Each treatment consists of five replicates. Means followed by the same letter within each column are not significantly different according to Tukey's test at $p < 0.05$.

Table 2. Two-way ANOVA analysis showing the effects of node position and PGRs on the average number of shoot production and shoot length from the responded explant.

Source of Variation	SS	df	Mean Square	F	*p* Value
No. of shoots					
Node position (N)	70.414	3	23.471	25.539	<0.001 **
PGRs (P)	88.621	6	14.770	16.071	<0.001 **
N × P	29.636	18	1.646	1.791	0.027 *
Shoot length					
Node position (N)	1.270	3	0.423	1.362	0.255 NS
PGRs (P)	49.240	6	8.207	26.397	<0.001 **
N × P	9.757	18	0.542	1.744	0.033 *

** *p* value is highly significant at $p < 0.001$ level, * significant at $p < 0.05$ level, NS—not significant.

3.3. Determination of Genetic Homogeneity Using ISSR

Five ISSR primers were utilized in this analysis, generating a total of 260 amplified DNA fragments ranging from 310 to 1200 bp (Figure 3). In this study, about 255 were monomorphic and 5 were polymorphic (Table 3). The percentage of monomorphism was 98.1% and polymorphism was 1.9%. Both primers of UBC 808 and UBC 836 produced 100% monomorphism, meanwhile the primer of UBC 835 produced one polymorphic band (1.5% polymorphism), and the primers of UBC 841 and UBC 842 produced two polymorphic bands with 3.9% and 3.7% polymorphism, respectively. The finding indicates

that the micropropagation of *M. limii* via nodal explants showed a low risk of genetic instability and therefore it is a reliable method for plantlet regeneration.

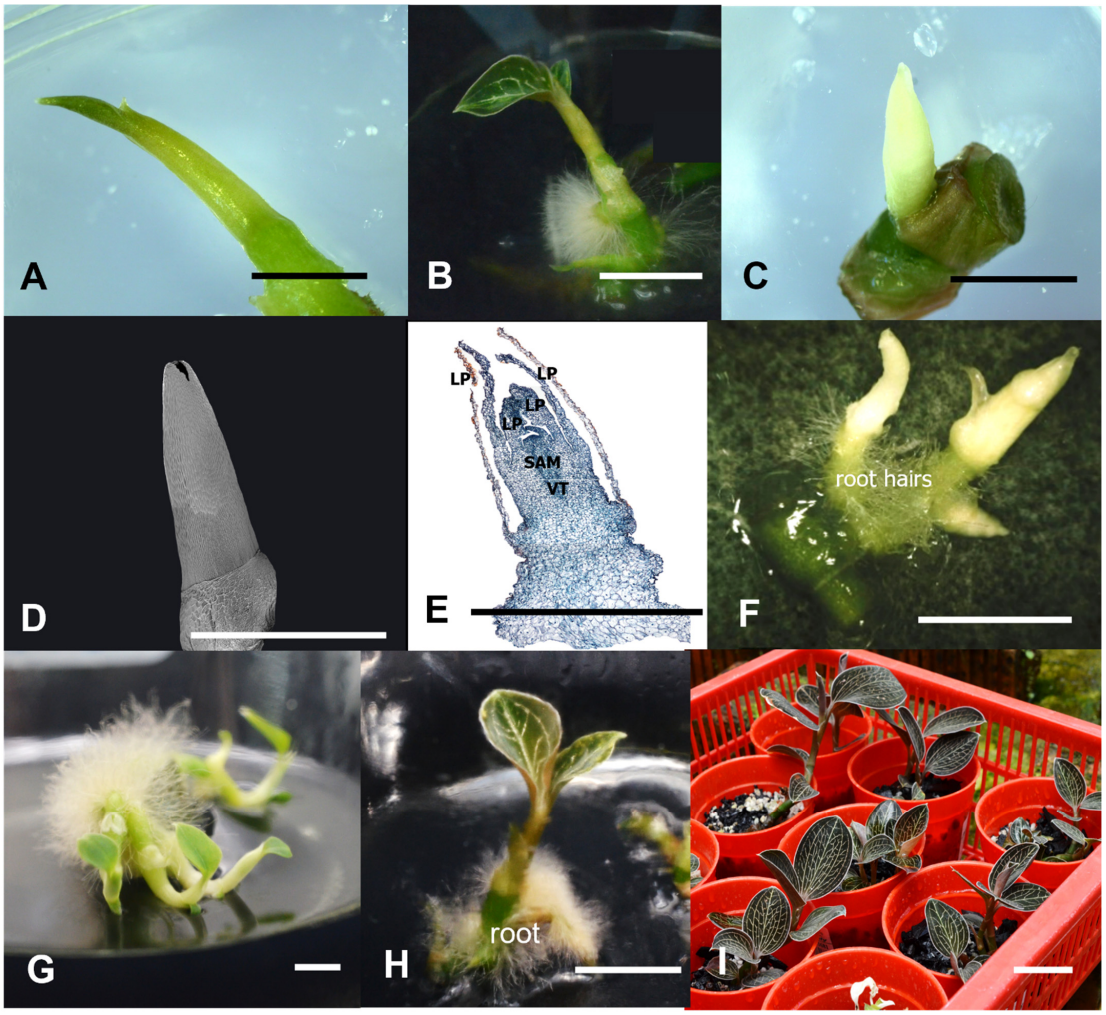

Figure 2. In vitro regeneration of *Macodes limii*. (**A**) Shoot elongated from the excised part of N1 (shoot tip) explant; (**B**) development of true leaves from shoot tip (N1) explant; (**C**) axillary bud break on N3 explant; (**D**) SEM of axillary bud on N3 explant; (**E**) histology of axillary shoot formation from N3 explant (LP—leaf primordia, SAM—shoot apical meristem, VT—vascular tissue); (**F**) multiple shoots induced from N3 explant; (**G**) multiple shoots with root hairs from N3 explant; (**H**) development of true leaves and root formation from the regenerated shoots; (**I**) fully acclimatized plantlets ready to be transferred to Poring Orchid Conservation Centre. Bars: (**A**–**G**) 0.5 cm; (**H**) 1 cm; (**I**) 3 cm.

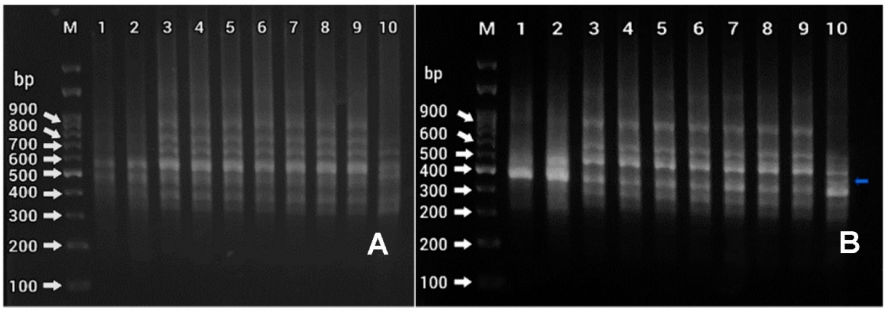

Figure 3. Gel electrophoresis of amplified fragments of *Macodes limii* produced by ISSR Primers (**A**) UBC 808 (**B**) UBC 835. Note: M: 100 bp marker (Fermentas); Lanes 1–2: mother plant; lanes 3–10: in vitro regenerated *M. limii*.

Table 3. List of primers and size of amplified fragments generated by ISSRs markers for DNA fingerprinting of in vitro plantlets of *M. limii*.

Primer Code	Primer Sequence (5′-3′)	Nucleotide Fragment Size (bp)	Total Bands Amplified	Monomorphic Band	Polymorphic Band	Monomorphism (%)	Polymorphism (%)
UBC808	AGAGAGAGAGA-GAGAGC	350–950	70	70	0	100	0
UBC835	AGAGAGAGAGA-GAGAGYC	325–1200	69	68	1	98.6	1.5
UBC836	AGAGAGAGAGA-GAGAGYC	600–1000	16	16	0	100	0
UBC841	GAGAGAGAGAGA-GAGAYC	320–900	51	49	2	96.1	3.9
UBC842	GAGAGAGAGAGA-GAGAYG	310–800	54	52	2	96.3	3.7
		Total	260	255	5	98.1	1.9

4. Discussion

Plant regeneration through shoot tip and nodal cultures has been reported to be the most applicable and reliable method of true-to-type in vitro propagation [12,33]. Different types of explants have different distributions of meristematic cells, which also differ among different species. In the present study, N2, N3, and N4 showed a significant response on shoot proliferation compared to N1 (shoot tip). The formation of axillary bud is controlled by apical dominance and endogenous hormones. Apical dominance is the process whereby the shoot tip inhibits the growth of axillary buds along the stem [34–36]. High production of auxin in shoot apex and subsequent basipetal auxin transport inhibits the outgrowth of axillary buds [37]. The removal of the shoot tip (apical meristem) caused lateral buds to break their dormancy and stimulated axillary bud outgrowth [38,39]. The suitability of nodal segment rather than shoot tip explant in the mass propagation of jewel orchids have been previously demonstrated in *A. sikkimensis*, *A. regalis* [40], and *A. elatus* [14]. Previously, Vasudevan and Staden [41] also reported that the absence of axillary shoot proliferation in shoot tip explants of *Ansellia africana* was due to auxin synthesized at the shoot tip explant, that exerted strong apical dominance. However, when compared to another study, the shoot tip explant was found superior to the nodal explant in axillary shoot regeneration of *A. formosanus* [16].

Cytokinins play many roles in plant development, often acting in concert with other hormones, most notably auxin, to regulate cell division and differentiation [42], apical dominance, and axillary bud outgrowth [43]. In this study, nodal explants of *M. limii* cultured on basal medium containing 1.0 mg/L of TDZ for 90 days yielded the highest number of axillary bud formations. TDZ possesses cytokinin- and auxin-like effects [44], which promoted the synthesis and/or accumulation of endogenous cytokinins, which significantly induced the proliferation of axillary shoots and released lateral buds from dormancy [45]. TDZ is widely used for micropropagation of many orchids including jewel orchids because of its incredible ability to induce organogenesis. TDZ has been previously used to induce axillary shoot production in jewel orchids including *A. formosanus* [46] and *A. elatus* [28,47]. TDZ, which is a substituted phenyl urea with cytokinin-like activity, has also been reported as an effective growth regulator for in vitro morphogenesis and organogenesis in other orchids including *Phalaenopsis* cv. Surabaya [48] and *Dimorphorcis lowii* [49].

The well-rooted plantlets of *M. limii* were acclimatized for two months, with a survival rate of 60%. The healthy acclimatized plantlets were successfully transferred to the conservation center for reintroduction. Plantlets produced in vitro should be gradually acclimatized to the ex vitro conditions to prevent high mortality after transfer to the environment of the field [50], and also to allow the plantlets to repair abnormalities in morphology, physiology, and anatomy before undergoing normal photosynthesis [51].

Typically, plantlets regenerated via axillary buds or direct somatic embryogenesis are considered to be the most genetically uniform [25,52]. The uniformity of the in vitro cultures against genetic variations despite repeated subcultures can be linked to their

respective genotype, which could be genetically resistant to alteration in their genetic materials [53]. However, artificial in vitro conditions, modified nutrients, and exposure to synthetic hormones might generate a genetic instability in cultured plants known as somaclonal variation [21,53,54]. Furthermore, the application of TDZ and prolonged exposure to it increased the chances of heritable somaclonal variation induction within the regenerated plantlets [55–57]. Molecular markers suitable for generating DNA profiles have served as an effective tool to assess the varietal identification, phylogenetic analysis, and genetic diversity [58]. In the current study, the genetic homogeneity of the in vitro propagated *M. limii* assessed via ISSR primers analysis, was 98.1% of monomorphism and 1.9% of polymorphism, indicating that the in vitro regenerated plants were confirmed as clonally uniform and genetically stable. Previously, the ISSR markers have been successfully utilized for the analysis of genetic fidelity within lines of some jewel orchids, including *A. elatus* [29] and *A. formosanus* [26].

5. Conclusions

Macodes limii is a Sabah endemic jewel orchid greatly appreciated for its attractive foliar venation but is still underexplored for its ornamental potential. Considering the rare and threatened status of the species, a protocol for in vitro regeneration of *M. limii* has been successfully established by using nodal explants, cultured on half-strength MS basal media incorporated with 1.0 mg/L TDZ. The in vitro regenerated plantlets showed high genetic similarity to the mother plant via an ISSR analysis, and were finally transferred to POCC for ex situ conservation. The current study has attempted to highlight the importance of the micropropagation protocol in combination with the reintroduction effort as an excellent opportunity for conserving endangered plant biodiversity and might as well be applied for large-scale propagation of this native plant.

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