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RESEARCH ARTICLE

Genetic fidelity assessment of micropropagated pepper (*Piper nigrum* L.) plantlets using ISSR markers

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ABSTRACT

Pepper (*Piper nigrum* L.) is a high-value spice crop, but in vitro propagation can induce somaclonal variation, necessitating genetic fidelity assessment. This study evaluated the genetic fidelity of micropropagated plantlets of a major Malaysian pepper cultivar Semongok Aman, generated via somatic embryogenesis using inter-simple sequence repeat (ISSR) markers. Tissue around the micropylar region in seeds, which were surface-sterilized and cultured under standardized conditions, was used to induce somatic embryogenesis. Twelve regenerated plantlets representing primary, secondary, and tertiary embryogenic stages, along with the field-grown mother plant, were analyzed. Genomic DNA was extracted and initially screened with 30 ISSR primers, of which six produced clear and reproducible amplification patterns and were selected for further analysis. These primers generated 64 scorable bands, comprising 54 monomorphic (84.4%) and 10 polymorphic (15.6%) bands. Genetic similarity between regenerated plantlets and the mother plant, calculated using the Jaccard coefficient, ranged from 0.902 to 1.000. Unweighted pair group technique with arithmetic average (UPGMA) cluster analysis grouped all regenerated plantlets with the mother plant at a 91% similarity threshold, while three clusters were resolved at a 95% threshold. Plantlets derived from primary and secondary somatic embryogenesis exhibited high genetic uniformity, whereas those originating from tertiary somatic embryogenesis displayed minor genetic variation. Overall, the findings demonstrate that somatic embryogenesis, particularly at primary and secondary stages, reliably produces true-to-type clones of 'Semongok Aman'. The optimized ISSR primers offer a robust and cost-effective tool for routine genetic fidelity assessment, supporting large-scale clonal propagation for sustainable pepper production in Malaysia and possibly in other pepper producing countries.

Key words: Genetic fidelity, ISSR markers, micropropagation, *Piper nigrum*, somatic embryogenesis.

INTRODUCTION

Pepper (*Piper nigrum* L.), widely known as the "king of spices" or "black gold", is one of the most economically important spice crops worldwide owing to its extensive use in the food, pharmaceutical, and cosmetic industries (Díaz-Guerrero et al., 2025). Belonging to the family Piperaceae, pepper is commercially processed as black or white pepper depending on the stage of harvest and whether the outer pericarp is retained (Sander et al., 2025). The crop has been utilized since ancient times and historically played a pivotal role in global trade, influencing the exploration of the New World during the 15th and 16th centuries. Its characteristic pungency is primarily

attributed to piperine (1-piperoyl-piperidine), a key biomarker used for standardizing raw pepper (*P. nigrum* and *P. longum*) and polyherbal formulations (Díaz-Guerrero et al., 2025).

Despite increasing global demand, pepper production remains constrained by labor intensive cultivation practices and high vulnerability to pests and diseases. Genetic improvement through breeding is challenging due to high levels of heterozygosity, polyploidy, and dioecy within the species (Khew et al., 2022). In this context, the integration of tissue culture techniques with breeding initiatives has proven an effective strategy for crop improvement across various species. However, in vitro regeneration systems may induce genetic variability, specifically somaclonal variation, which can be either epigenetic or heritable in nature. Such variation may adversely affect clonal uniformity, yield, and quality in commercially important crops, including pepper (Ferreira et al., 2023).

Consequently, ensuring genetic uniformity of regenerated pepper plantlets is essential for their commercial application, and early assessment of genetic fidelity is critical. Molecular markers, particularly polymerase chain reaction (PCR)-based assays, have been widely employed to monitor genetic stability. Among these, inter-simple sequence repeat (ISSR) markers are favored for their simplicity, cost-effectiveness, reproducibility, and independence from prior sequence information (Tippani et al., 2025). While ISSR markers have been extensively utilized in plant genetic studies (Hancı and Paşazade, 2025), their application in assessing the genetic fidelity of Malaysian pepper plantlets has not been reported.

To the best of our knowledge, this study presents the first systematic evaluation of genetic fidelity in micropropagated 'Semongok Aman', a main local pepper cultivar, using ISSR markers. Clonal plantlets were generated through an optimized and reproducible somatic embryogenesis protocol, and genetic stability was evaluated across distinct embryogenic stages. By linking regeneration strategy and embryogenic stage with molecular stability, this work addresses a critical gap in pepper micropropagation research and establishes a practical framework for quality assurance in large-scale clonal propagation. The findings support the development of reliable propagation systems for sustainable pepper production in Malaysia and possibly beyond.

MATERIALS AND METHODS

Plant material and explant preparation

Fully ripe and mature berries of a major Malaysian pepper (*Piper nigrum* L.) cultivar, Semongok Aman, were collected from a commercial pepper farm in the Balai Rigin District (approximately 1.049° N, 110.796° E), Sri Aman Division, Sarawak, Malaysia. The berries were washed with laboratory detergent and gently rubbed on a stainless-steel sieve to remove pericarps, followed by continuous rinsing under running tap water for 1 h. Tissue around the micropylar region of pepper seed was used as the explant to initiate somatic embryogenesis. Under aseptic conditions, the seeds were immersed in 0.3% (w/v) benomyl solution for 20 min and 96% (v/v) ethanol for 1 min. Further surface sterilization was conducted in a laminar airflow cabinet using four different treatments: Soaking in either 30% or 35% (v/v) of Clorox solution containing 5.25% sodium hypochlorite and 0.1% (v/v) of Tween-20 solution for 15 or 20 min with constant and gentle agitation. Following sterilization, explants were rinsed 5 times for 5 min with sterile distilled water and surface-dried on autoclaved filter paper under sterile conditions.

Plant regeneration and acclimatization

Sterilized explants were cultured on Murashige and Skoog (MS) and Schenk and Hildebrandt (SH) basal medium supplemented with 30 g L^{-1} sucrose, 3 g L^{-1} polyvinylpyrrolidone, and solidified with 3.0 g L^{-1} gelrite. The pH of the media was adjusted to 5.8 prior to autoclaving at 121 °C and 1.05 kg cm^{-2} for 20 min. Cultures were incubated in a growth chamber at 25 ± 1 °C in complete darkness and sub-cultured monthly onto fresh medium of the same composition (Parab et al., 2021). After 4 mo culture, media were supplemented with neutralized activated charcoal. Morphological changes were documented at regular intervals.

Well-developed plantlets with healthy root systems were carefully removed from the media and rinsed under running tap water to remove adhering media. To prevent fungal contamination, plantlets were treated with 0.1% (w/v) benomyl for 30 min before being transferred to polythene bags containing a sterile mixture of compost, soil, sand, and vermiculite (1:1:1:1 v/v/v/v). Transplanted plantlets were maintained in laboratory plant propagators for 21 d to ensure high humidity and protection from mechanical damage. Subsequently, the

plantlets were gradually transferred to a screen-shaded plant house and irrigated regularly following the emergence of new leaves and shoots. Survival rate (%) was calculated 1 mo after transplantation using the following equation (Chen et al. 2024):

$$\text{Survival rate (\%)} = \frac{\text{Number of plants showing new growth}}{\text{Total number of plants acclimatized}} \times 100\%$$

DNA extraction and PCR amplification

Genetic fidelity of micropropagated plantlets was assessed using inter-simple sequence repeat (ISSR) markers. Young leaf samples were obtained from field-grown 'Semongok Aman' mother plants and from 12 randomly selected somatic embryogenesis-derived plants grown at the Malaysian Pepper Board (MPB), Kuching, Sarawak, Malaysia. The micropropagated plants originated from different stages of somatic embryogenesis, including primary (SE1-SE8), secondary (SE9-SE10 and SE12), and tertiary (SE11) cycles. Additionally, 'Kuching' (K) served as a reference control to verify the ability of the ISSR markers to differentiate among genetically distinct genotypes. Total genomic DNA was extracted using the Genomic DNA Purification Kit (Wizard, Promega, Madison, Wisconsin, USA), following manufacturer's protocols with minor modifications. The DNA concentration and purity were determined using the Gene-Quant 1300 UV spectrophotometer (GE Healthcare, Chicago, Illinois, USA) by measuring absorbance at 260 and 280 nm with a dilution factor of 100.

A total of 30 commercially available ISSR primers were initially screened, and only primers producing clear, reproducible banding patterns were selected for further analysis (Table 1). The PCR program consisted of an initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 43 to 49 °C for 2 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplified products were separated on 1.8% (w/v) agarose gels containing 1X SYBR Safe DNA gel stain in 1× TAE buffer for 4 h. The ISSR-PCR protocol in this study was further optimized for the selected primers by varying $MgCl_2$ (1.5, 2.0, and 2.5 mM) and DNA template (10, 15, and 20 ng μL^{-1}) concentrations.

Table 1. Optimized ISSR primers with their annealing temperatures and reaction mixtures.

Primer	Primer sequence (5'-3')	Size range	Monomorphic bands	Polymorphic bands	Annealing temperature	$MgCl_2$	DNA
		bp			°C	mM	ng μL^{-1}
ISSR-807	$(AG)_8T$	300-1300	8	3	49	1.5	10
ISSR-808	$(AG)_8C$	400-2000	10	1	47	1.5	20
ISSR-809	$(AG)_8G$	400-2000	11	1	43	1.5	10
ISSR-812	$(GA)_8A$	500-1300	6	2	45	2.5	15
ISSR-816	$(CA)_8T$	500-1500	9	1	49	2.0	10
ISSR-830	$(TG)_8G$	400-2000	10	2	47	2.5	10

The sample size included all selected genotypes under investigation, which were considered sufficient to capture the genetic variability relevant to the study objectives. To ensure the reliability and reproducibility of the molecular data, DNA was extracted independently from three biological replicates per genotype, and each PCR amplification was performed in triplicate. Only bands that were consistently reproduced across replicate amplifications were scored for subsequent analyses. Gels were visualized using the Safe Imager 2.0 Blue-Light Transilluminator (Invitrogen, Waltham, Massachusetts, USA) and documented using the AlphaImager HP Gel Documentation System (ProteinSimple, Santa Clara, California, USA).

Data analysis

Gel images were assessed based on reproducible and well-resolvable bands using band-to-band scoring. The DNA banding profiles of micropropagated plantlets were compared with those of the field-grown mother plants. Bands that were faint or ambiguous were excluded from analysis. Clear bands were scored as present (1) or absent (0) (Abd-Elsamei et al., 2025). The resulting binary data matrix was analyzed using FreeTree software version

0.9.1.50 (Pavlíček et al., 1999). Genetic similarity among samples was estimated using the Jaccard similarity coefficient. A dendrogram was constructed based on similarity coefficients using the unweighted pair group technique with arithmetic average (UPGMA) (Elgohary et al., 2025) and visualized with TreeView software version 1.6.5. (Page, 1996).

RESULTS

To optimize surface sterilization of Malaysian pepper explants, four treatments differing in sodium hypochlorite (Clorox, commercial bleach) concentration and soaking duration were evaluated, as described in the methodology. Among the treatments, sterilization with 35% Clorox (5.25% NaOCl) with 0.1% (v/v) Tween-20 for 20 min was most effective, significantly reducing microbial contamination and yielding 82% aseptic cultures.

A total of 30 ISSR primers were screened in the current study of which six primers produced clear, reproducible profiles and were subsequently selected for further analysis (Table 1). Of these, 64 distinct bands were generated, with an average of 10.67 scorable bands per primer (Table 1; Figure 1). Among the 64 bands, 54 were monomorphic in the micropropagated plantlets, while 10 bands (15.6%) were polymorphic (Table 1). The optimum annealing temperature for ISSR markers ranged from 43.0 to 49.0 °C (Table 2). The ISSR-PCR protocol in the present study was further optimized for the selected primers by evaluating $MgCl_2$ concentrations of 1.5, 2.0, and 2.5 mM, as well as DNA template concentrations of 10, 15, and 20 ng μL^{-1} (Table 1). The selected PCR conditions produced clear and consistent amplification profiles across samples.

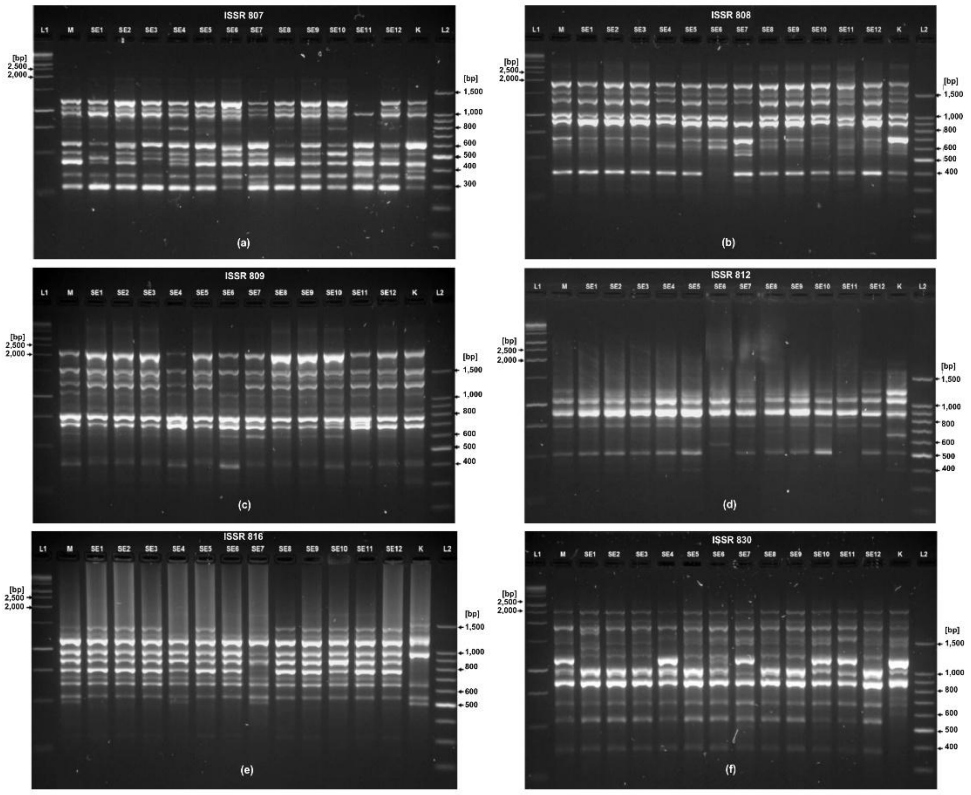

Figure 1. Amplified products using primers ISSR-807 (a), ISSR-808 (b), ISSR-809 (c), ISSR-812 (d), ISSR-816 (e), and ISSR-830 (f). L1: 1 kb DNA ladder; M: 'Semongok Aman' (mother plant); SE1-12: micropropagated plantlets; K: 'Kuching' (control); L2: 100 bp DNA ladder.

Table 2. Similarity matrix of the field-grown mother plant of 'Semongok Aman' (M), micropropagated plantlets (SE), and 'Kuching' (K) measured by the Jaccard's similarity coefficient.

	M	SE1	SE2	SE3	SE4	SE5	SE6	SE7	SE8	SE9	SE10	SE11	SE12	K
M	1.000													
SE1	0.952	1.000												
SE2	0.967	0.983	1.000											
SE3	0.952	1.000	0.983	1.000										
SE4	0.984	0.935	0.951	0.935	1.000									
SE5	0.967	0.983	1.000	0.983	0.951	1.000								
SE6	0.921	0.905	0.919	0.905	0.905	0.919	1.000							
SE7	0.968	0.921	0.935	0.921	0.952	0.935	0.921	1.000						
SE8	0.967	0.983	1.000	0.983	0.951	1.000	0.919	0.935	1.000					
SE9	0.967	0.983	1.000	0.983	0.951	1.000	0.919	0.935	1.000	1.000				
SE10	0.984	0.967	0.983	0.967	0.967	0.983	0.935	0.951	0.983	0.983	1.000			
SE11	0.918	0.902	0.917	0.902	0.933	0.917	0.902	0.918	0.917	0.917	0.933	1.000		
SE12	0.967	0.983	1.000	0.983	0.951	1.000	0.919	0.935	1.000	1.000	0.983	0.917	1.000	
K	0.761	0.746	0.758	0.746	0.746	0.758	0.696	0.761	0.758	0.758	0.746	0.712	0.758	1.000

Cluster analysis was performed using similarity coefficients derived from ISSR marker data based on 64 scored bands (Figure 1; Table 1). Similarity indices between the mother plant and the micropropagated plants ranged from 0.902 to 1.00, indicating a low level of somaclonal variation within the micropropagated population (Table 2). The UPGMA dendrogram revealed that all 12 micropropagated plants clustered together as a single group with 91% similarity. Only at a more stringent similarity threshold of 95% were three distinct clusters resolved (Figure 2). It is worth noting that the plantlets analyzed represented different somatic embryogenesis stages, including primary (SE1-SE8), secondary (SE9-SE10 and SE12), and tertiary (SE11).

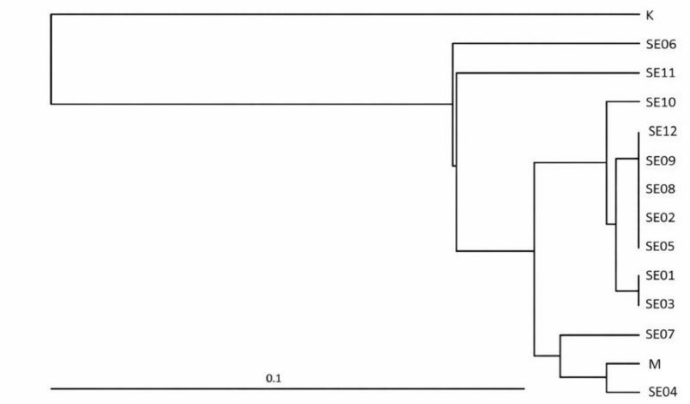

Figure 2. Unweighted pair group technique with arithmetic average (UPGMA) dendrogram of the genetic relationship between field-grown mother plant of 'Semongok Aman' (M), micropropagated plantlets (SE), and 'Kuching' (K) constructed using TreeView program.

DISCUSSION

The establishment of axenic cultures from field-grown pepper is constrained by endophytic microbial contamination and the release of phenolic compounds, both of which adversely affect tissue viability (Gunawardhana et al., 2022). The present study achieved a high aseptic culture rate of 82%, highlighting the effectiveness of the sterilization protocol employing 35% sodium hypochlorite (5.25% NaOCl) in combination with 0.1% (v/v) Tween-20 for 20 min. Similar observations have been reported by Hancı and Paşazade (2025), who demonstrated the importance of optimizing sterilant concentration and exposure time to balance

contamination control and tissue survival. Considering that excessive exposure to disinfectants can impair explant regeneration, the use of aseptically propagated seedlings as explant sources is generally recommended to maximize morphogenic response and regeneration efficiency (Wang et al., 2025).

Maintaining genetic uniformity in micropropagated plants is essential, yet tissue culture can induce somaclonal variation. Hence, assessing genetic fidelity is critical for ensuring the consistency and commercial viability of regenerated plants (Ferreira et al., 2023). The ISSR markers are widely used to evaluate clonal fidelity in tissue culture-derived plants (Hancı and Paşazade, 2025). The predominance of monomorphic bands observed in this study (Table 1) indicates that somatic embryogenesis largely maintained the genetic integrity of regenerated plantlets. This demonstrates that ISSR markers are effective for assessing genetic variation between the field-grown 'Semongok Aman' mother plant and its somatic embryogenesis-derived progenies, consistent with recent reports in other plant species such as *Juniperus procera* Hochst. ex Endl. (Al-Yasi and Al-Qthanin, 2024) and *Pterocarpus marsupium* Roxb. (Tippani et al., 2025).

The optimum annealing temperatures for the selected ISSR primers ranged from 43.0 to 49.0 °C (Table 2). Optimization of PCR conditions is fundamental for generating reproducible ISSR profiles. Excess $MgCl_2$ may promote non-specific primer annealing, whereas insufficient $MgCl_2$ can reduce DNA polymerase activity. Similarly, inappropriate template DNA concentrations can result in unreliable banding patterns (Munankarmi et al., 2022). The high similarity coefficients and clustering pattern observed in the UPGMA analysis further support the genetic stability of the regenerated plants (Figure 2). High levels of genetic similarity are desirable in commercial propagation programs, where the production of true-to-type planting materials is essential for maintaining uniform agronomic performance and quality traits (Ibrahim et al., 2024). Similar observations have been reported in other micropropagated crops, including apple (Bisht et al., 2024), where ISSR-based cluster analyses revealed close genetic relationships between regenerants and their mother plants, confirming minimal somaclonal variation induced during in vitro culture.

Additionally, our results indicate that micropropagation of 'Semongok Aman' via somatic embryogenesis is most reliable when limited to primary and secondary cycles. Tertiary somatic embryogenesis is not recommended, as it may increase the risk of genetic variation or instability, potentially due to naturally occurring variation or the accumulation of mutations during prolonged in vitro culture (Duta-Cornescu et al., 2023). Overall, the results demonstrate that the somatic embryogenesis protocol used in this study is suitable for the large-scale propagation of genetically stable pepper plants, while highlighting the utility of ISSR markers as an effective molecular tool for quality control in micropropagation programs.

CONCLUSIONS

This study demonstrated the effectiveness of ISSR markers in assessing the genetic fidelity of somatic embryogenesis-derived pepper plants relative to their field-grown mother plant. The uniform banding patterns observed among regenerated plantlets indicated the genetic stability of the micropropagation protocol and supported the use of ISSR markers as a reliable tool for clonal fidelity assessment. These findings reinforce the potential of somatic embryogenesis for large-scale propagation of true-to-type pepper planting materials. However, the study was limited by the relatively small sample size of 12 regenerated plantlets, and future studies involving larger populations and complementary molecular markers would provide a more comprehensive evaluation of genetic stability.

Author contribution

Conceptualization: K.P.E., N.A.R., N.K., S.S.L., A.C. Methodology: K.P.E., N.A.R., N.K., A.C. Formal analysis: K.P.E. Writing-original draft: K.P.E., A.C. Writing-review & editing: All co-authors. Supervision: N.A.R., N.K., A.C. Project administration: N.A.R., N.K. Funding acquisition: K.P.E., A.C. All co-authors reviewed the final version and approved the manuscript before submission.

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