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ORIGINAL ARTICLE

Molecular Assessment of Clonal Fidelity in *In Vitro*-Regenerated *Heinsia crinita* (African bush apple) Micro Shoots Using ISSR and SCoT markers

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ARTICLE INFO

Received: 1st May 2026
Revised: 26th August 2026
Accepted: 29th August 2026
Published online: 31st August 2026
DOI:https://dx.doi.org/10.4314br.v24i2.15

e-ISSN: 2705-3822
print-ISSN: 1596-7409

Official. Faculty of Biological Sciences, University of Nigeria.

ABSTRACT

The African bush apple *Heinsia crinita* (Wennberg) G. Taylor is a valuable orphan shrub with important nutritional and medicinal uses, especially in southern Nigeria. To support germplasm conservation and biotechnology-driven improvement, we previously developed the first *in vitro* regeneration system for the crop using stem and hypocotyl explants. In this study, genetic stability was assessed in ten *in vitro* regenerants, comprising five stem-derived and five hypocotyl-derived microshoots, using 10 Inter-Simple Sequence Repeat (ISSR) and 3 Start Codon-Targeted (SCoT) primers selected for clear and reproducible amplification. Using ISSR primers, 38 reproducible bands (250 ~ 2200 bp) were amplified, and microshoots regenerated from stem and hypocotyl explants showed 5.26% and 0.00% loci polymorphism, respectively. The SCoT primers produced 6 reproducible bands (150–2100 bp) and showed 0.00% polymorphism across both explant types. The polymorphism assessment was based on the presence or absence of clear, reproducible bands across the regenerants relative to the mother plant. Similarity coefficients ranged from 0.955 to 1.000, and UPGMA clustering grouped the regenerants into one major cluster with two minor sub-groupings. The mother plant clustered with all hypocotyl-derived microshoots and most stem-derived microshoots, indicating a high degree of apparent genetic similarity. Overall, hypocotyl-derived regenerants showed slightly greater stability in this dataset, however further validation using additional markers as well as larger sample sizes, would strengthen the recommendation.

Keywords: African bush apple; *Heinsia crinita*; Genetic homogeneity; Molecular markers.

Cite this article: Chukwurah, P. N., Okosi G. M., Akuoma, O. M., Ojong, M. S., Chineke, D. O., Haruna D. Y., & Okeke, P. C. (2026). Molecular assessment of clonal fidelity in *in vitro*-regenerated *heinsia crinita* (African bush apple) micro shoots using ISSR and SCoT markers. *Journal of Biological Research and Biotechnology,* 24(2), 625-631.
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DOI: https://dx.doi.org/10.4314br.v24i2.15

1.0 INTRODUCTION

The African bush apple [*Heinsia crinita* (Wennberg) G. Taylor] is a perennial shrub of the *Rubiaceae* family native to tropical areas of Central and West Africa. In Nigeria, it is particularly endemic in the humid southern region where the leaves are primarily used as a vegetable in local soups but also in ethnomedicine. Nutritional and phytochemical analysis of *H. crinita* leaves and fruits showed they are rich in protein, fiber, essential minerals, phenols, flavonoids, saponins, tannins, glycosides and terpenoids (Ekpo et al.., 2012; Morah & Ashipu, 2017; Divine-Anthony et al., 2022). *H. crinita* remains a neglected and underutilized species (NUS), despite the considerable promise of NUS for food and health security in sub-Saharan Africa (Chivenge et al., 2015). To ensure a sustainable production and conservation of the crop and reduce pressure on wild populations, its first direct *in vitro* shoot regeneration protocol was published by Chukwurah et al. (2024), achieving high shoot proliferation from stem and hypocotyl explants with over 90% acclimatization success. However, *in vitro* propagation methods often introduce genetic defects caused by somaclonal variation, which can result in genotypic and phenotypic changes that may affect plant integrity, medicinal quality, nutritional value, clonal uniformity, and other desirable traits (Ferreira et al., 2023). For *H. crinita*, this is particularly important because any culture-induced variation could compromise the consistency of traits linked to its nutritional, ethnomedicinal, and conservation value, thereby limiting the reliability of micropropagated plants for germplasm preservation, domestication, and future pharmaceutical use. According to Lakshmanan et al. (2007), such somaclonal variations may occur due to the type of explant used, the influence of plant growth regulators, as well as repeated subcultures during micropropagation. Genetic fidelity testing is, therefore, important to ensure genetic stability of clonally propagated plants relative to the parent plant, and dominant molecular markers such as Inter Simple Sequence Repeats (ISSRs) and Start Codon-Targeted (SCoT) provide a reliable and effective method for assessing genetic variation and detecting polymorphisms.

The ISSR marker system uses single microsatellite sequences as primers in PCR to generate multi-locus markers (Avinash & Rajarsh, 2020), while Start Codon Targeted (SCoT) markers detect variation by targeting the short regions flanking the "ATG" start codon in plant genes which are usually conserved across plant species (Collard & Mackill, 2009). Both ISSR and SCoT segregate as dominant markers following simple mendelian inheritance (Yadav, 2023) and can be measured quickly and efficiently without prior genetic sequence information (Haidar et al., 2021). These features make them particularly suitable for orphan crops such as *H. crinita*, for which genomic resources remain limited and no species-specific co-dominant marker set is yet available. Compared with marker systems such as SSRs or SNP-based approaches, ISSR and SCoT require no prior sequence development, are cost-effective, and can generate informative multilocus profiles across regenerated plantlets. They also offer greater reproducibility than RAPD markers, making them a practical choice for preliminary clonal fidelity assessment in under-studied species.

Given the importance and potential of *H. crinita* for food security and health **in** sub-Saharan Africa, and given that no prior study has assessed the genetic stability of its *in vitro* regenerants, this study was aimed at unraveling the extent to which its regenerated micro shoots genetically resemble the mother plant. Accordingly, we used ISSR and SCoT marker systems to evaluate and compare genetic fidelity of the regenerants from *H. crinita*'s stem and hypocotyl explants produced from our previous study. This work provides the first molecular assessment of clonal fidelity in ***H. crinita*** and fills an important gap in the biotechnology of this neglected species. By establishing the extent to which regenerated plantlets retain the genetic profile of the mother plant, the study offers a foundation for reliable large-scale micropropagation, *ex situ* germplasm conservation, and the development of improved propagation systems for domestication efforts. More broadly, the findings contribute to the growing body of knowledge needed to support molecular breeding, conservation, and biotechnological improvement of *H. crinita* and other underutilized African crops with similar genomic limitations.

2.0 MATERIALS AND METHODS

2.1 Extraction of genomic DNA from in vitro regenerated micro shoots

In our previous study (Chukwurah et al., 2024), we used combinations of two cytokinins (benzyl adenine – BA, thidiazuron – TDZ) and the auxin (naphthalene acetic acid (NAA) to develop an *in vitro* regeneration system for *H. crinita* based on direct shoot proliferation from stem and hypocotyledonary explants (Figure 1). Murashige and Skoog (MS) medium was used. Here, ten (10) of the micro shoots regenerated from the stem and hypocotyl explants were randomly selected for the assessment of clonal fidelity. Five of the micro shoots were randomly selected from independently regenerated microshoots of each explant type, so that the samples represented biological replicates from different explant sources rather than repeated selections from a single explant lineage. 100 mg fresh leaf sample was taken from each selected shoot as well as from 4-week-old mother plant (germinated *in vitro* on Murashige and Skoog (MS) medium containing 1% sucrose, 0.5 g/L 2-(N-morpholino) ethane sulfonic acid (MES), and 0.8% agar, pH 5.7), and used for genomic DNA extraction using the standard cetyltrimethylammonium bromide (CTAB) method (Peredo, 2023). DNA quality and quantity were assessed using a NanoDrop spectrophotometer before used for downstream PCR amplification.

2.2 Optimization of ISSR/SCoT primers and PCR amplification of markers *loci*

Ten ISSR primers (developed by the University of British Columbia (UBC), Vancouver, Canada), and five SCoT primers were selected, synthesized, and optimized via PCR reactions to determine the annealing temperatures that yield clear and reproducible bands. After optimization, 10 ISSR and 3 SCoT primers (Table 1) were selected for subsequent PCR amplification of ISSR and SCoT markers in the samples. PCR reactions were carried out in a 20 µL cocktail (10 µL of 2x PCR buffer, 4 µL of 2 mM dNTPs, 1 µL of 10 pmol ISSR/SCoT primer, 1 µL of 50 ng genomic DNA, 0.4 µL of KOD Fx Neo polymerase, 3.6 µL nuclease-free water) using the KOD Fx Neo PCR enzyme kit system (TOYOBO, Japan). Each amplification run included a negative control containing the complete reaction mixture except template DNA to monitor contamination. All PCR amplifications were performed in triplicate to confirm reproducibility.

Table 1: ISSR and SCoT primer sequences used in the study

Primer name	Sequence (5′ - 3′)	Anchor	Annealing Temp. (°C)
UBC 841	$(GA)_8YC$	3′ anchor	55
UBC 848	$(CA)_8RG$	3′ anchor	52
UBC 818	$(CA)_8G$	3′ anchor	52
UBC 826	$(AC)_8C$	3′ anchor	55
UBC 815	$(CT)_8G$	3′ anchor	55
UBC 810	$(GA)_8T$	3′ anchor	55
UBC 807	$(AG)_8T$	3′ anchor	55
UBC 856	$(AC)_8YA$	3′ anchor	55
UBC 824	$(TC)_8G$	3′ anchor	52
UBC 873	$(GACA)_4$	unanchored	55
SCoT 1	CAACAATGGCTACCACCA	-	54
SCoT 6	ACGACATGGCGACCAACG	-	55
SCoT 10	CACCATGGCTACCACCAG	-	55

R = (A, G); Y = (C, T); ATG = Start Codon. Cycling conditions were programmed as: one cycle of pre-denaturation (94°C for 3 mins), 30 cycles of denaturation-annealing-extension (98°C for 10 seconds, 52/54/55°C for 30 seconds, and 68°C for 60 seconds), and one cycle of final extension was set at 68°C for 5 mins. All PCR amplifications were carried out thrice to ensure reproducibility of marker bands before scoring.

2.3 Scoring and analysis of ISSR/SCoT marker bands/loci

Following amplification with each ISSR and SCoT primer, the PCR products were separated on 1.3% agarose gels stained with ethidium bromide and only clearly distinctive bands with strong intensities within a standard size range of 150 - 2200 bp were scored and recorded in a binary form: 1 for presence and 0 for absence. Each scored ISSR or SCoT band was regarded as a locus and for each primer, total number of unique bands, number of polymorphic OR monomorphic bands, and percentage of polymorphic/monomorphic bands were analyzed using regenerants of each explant type. Polymorphic bands were defined as unique ISSR or SCoT bands present in some samples but absent in others, whereas monomorphic bands were those present in all samples. The percentage of polymorphic OR monomorphic bands was calculated as the ratio of number of polymorphic OR monomorphic bands to the total number of bands multiplied by 100).

2.4 Genetic analysis of scored marker loci

The binary scores generated were used to compute a genetic similarity matrix among the samples. A dendrogram constituted based on Unweighted Pair Group Means Average (UPGMA) was used to estimate the degree of genetic similarity of micro shoots regenerated from both stem and hypocotyl explants to the mother plant. All genetic analysis was done using the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc) version 2.2 software.

3.0 RESULTS

3.1 Analysis of scorable ISSR and SCoT markers

ISSR primers revealed a total of 38 distinct and scorable bands, ranging from 250 bp to 2200 bp, across the mother plant and the regenerants from stem (S1–S5) and hypocotyl (H1–H5) explants (Table 2). Of these, 36 bands were monomorphic across all samples while 2 were polymorphic,, giving an overall monomorphism rate of 94.74%. The polymorphism was detected only in stem-derived regenerants, whereas all hyptocotyl-derived regenerants were fully monomorphic across the ISSR loci scored. Detailed primer-wise banding patterns are presented in Table 2 and Figure 1a and S2. The SCoT primers amplified six (6) distinct bands across the 11 samples, with fragment sizes ranging from 150 bp to 2100 bp. All six bands were monomorphic across the mother plant, stem-derived regenerants, and hypocotyl-derived regenerants, indicating uniformity at the SCoT loci analyzed (Table 2 and Figure 1). Compared with ISSR, the SCoT analysis showed no detectable polymorphism in either explant type.

3.2 Genetic analysis of scored marker bands

The genetic relatedness of the mother plant (Mo) and *in vitro* regenerants was estimated using a similarity index matrix based on simple matching coefficients (Table 2). The analysis high apparent genetic similarity among the samples, with coefficients ranging from 0.955 to 1.000. The mother plant (Mo) showed a similarity coefficient of 1.000 with all hypocotyl-derived shoots (H1 – H5) as well stem-derived shoots S2, S3,

S4. Slight divergence was observed in stem-derived regenerants S1 and S5, which still shared high similarity coefficients of 0.977 with the mother plant. The UPGMA dendrogram (Figure 1d) grouped the samples into one major cluster and two minor offshoots. The mother plant (Mo) clustered tightly with stem-derived regenerants S2, S3, S4, as well as the entire hypocotyl-derived regenerants (H1–H5), while S1 and S5 formed distinct branches that remained closely linked to the main cluster at coefficients of approximately 0.98 and 0.97, respectively. These small deviations likely reflect minor explant-related or culture-associated differences rather than substantial genomic divergence.

Figure **1.** (A) *H. crinita* plant (B) Fresh *H. crinita* fruits (C) Dry *H. crinita* fruits (D) Hypocotyl and stem explants of *H. crinita* used for *in vitro* regeneration experiments in Chukwurah *et al*. (2024) (E) *H. crinita* micro shoots regenerated from hypocotyl and stem explants

Table **2.** Analysis of scorable bands (loci) amplified in *H. crinita* regenerants using ISSR and SCoT primers

Primer name	Number of unique bands amplified*	Range of band sizes (bp)	No. polymorphic bands		No. monomorphic bands		% polymorphic bands		% monomorphic bands	
			stem	*hypocotyl*	*stem*	*hypocotyl*	*stem*	*hypocotyl*	*stem*	*hypocotyl*
UBC 841	6	300 – 2200	1	0	5	5	16.7	0.0	83.3	100.0
UBC 848	2	250 – 750	0	0	2	2	0.0	0.0	100.0	100.0
UBC 818	2	600 – 900	0	0	2	2	0.0	0.0	100.0	100.0
UBC 826	4	500 – 850	0	0	4	4	0.0	0.0	100.0	100.0
UBC 815	3	650 – 1500	0	0	3	3	0.0	0.0	100.0	100.0
UBC 873	2	1200 – 2000	1	0	1	1	50.0	0.0	50.0	100.0
UBC 810	4	700 – 1700	0	0	4	4	0.0	0.0	100.0	100.0
UBC 807	7	350 – 1500	0	0	7	7	0.0	0.0	100.0	100.0
UBC 856	3	700 – 900	0	0	3	3	0.0	0.0	100.0	100.0
UBC 824	5	600 – 1900	0	0	5	5	0.0	0.0	100.0	100.0
Total	38	250 – 2200	2	0	36	36	5.26	0.0	94.74	100.0
SCoT 1	1	2100	0	0	1	1	0.0	0.0	100.0	100.0
SCoT 6	3	150 – 380	0	0	3	3	0.0	0.0	100.0	100.0
SCoT 10	2	1200 – 2000	0	0	2	2	0.0	0.0	100.0	100.0
Total	6	150 – 2100	0	0	6	6	0.0	0.0	100.0	100.0

**Only unique bands of 150 ~ 2200 bp that showed up in at least two repeated PCR were scored and used in further analysis. UBC: University of British Columbia; SCoT: Start codon-targeted*

Table **3.** Similarity index matrix showing the genetic similarity between mother plant (Mo) and *in vitro* regenerants (S1 - H5) based on simple matching coefficients.

	Mo	S1	S2	S3	S4	S5	H1	H2	H3	H4	H5
Mo	1.000										
S1	0.977	1.000									
S2	1.000	0.977	1.000								
S3	1.000	0.977	1.000	1.000							
S4	1.000	0.977	1.000	1.000	1.000						
S5	0.977	0.955	0.977	0.977	0.977	1.000					
H1	1.000	0.977	1.000	1.000	1.000	0.977	1.000				
H2	1.000	0.977	1.000	1.000	1.000	0.977	1.000	1.000			
H3	1.000	0.977	1.000	1.000	1.000	0.977	1.000	1.000	1.000		
H4	1.000	0.977	1.000	1.000	1.000	0.977	1.000	1.000	1.000	1.000	
H5	1.000	0.977	1.000	1.000	1.000	0.977	1.000	1.000	1.000	1.000	1.000

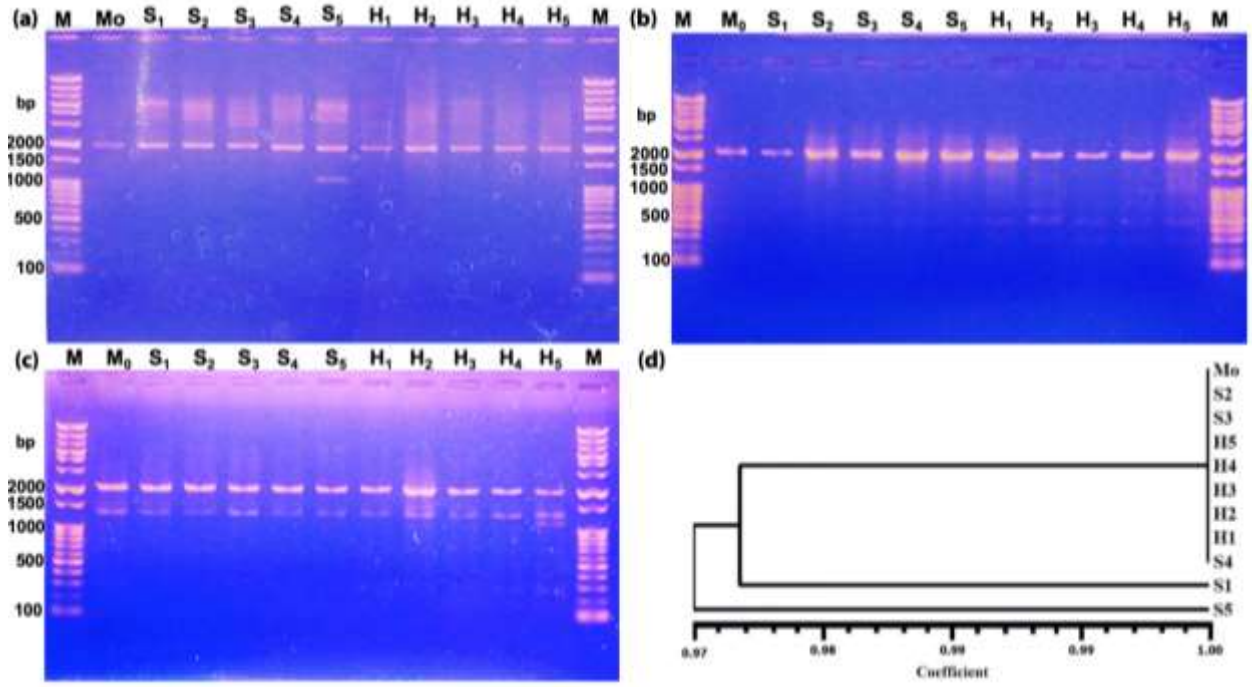

Figure ***2.*** *PCR amplification of ISSR/SCoT marker loci in H. crinita and cluster analysis of H. crinita samples. Electrophoretic profiles of H. crinita mother (Mo) and in vitro regenerants showing marker bands/loci amplified using (a) UBC 873 (b) SCoT 1 (c) SCoT 10 primers. (d) UPGMA clustering dendrogram showing the relationship between mother H. crinita plant (Mo) and in vitro regenerants. M: Gene ladder wide 2 (0.1 – 20 kbp); Mo: Mother plant; $S_1 – S_5$: regenerants derived from shoot explants; $H_1 – H_5$: regenerants derived from hypocotyl explants.*

4.0 DISCUSSION

ISSR marker analysis generally revealed a high degree of apparent genetic uniformity between the mother plant and the regenerants, though a comparative look at the explant types revealed slight differences. While the hypocotyl-derived regenerants exhibited 100% monomorphism across the ISSR loci analyzed, this should be interpreted as high apparent genetic fidelity rather than absolute genetic stability, especially given the limited marker resolution of dominant ISSR markers. Stem-derived regenerants, in contrast, showed a 94.74% monomorphism rate, with only two primers (UBC 841 and UBC 873) detecting slight polymorphisms in some micro shoots. These findings are broadly consistent with studies on woody and medicinal plants where ISSR markers indicated high clonal fidelity. For instance, Bisht et al. (2024) reported 98.43% ISSR monomorphism in *in vitro* regenerated apple rootstock, while Negi & Saxena (2010) similarly reported high retention of monomorphism in *Bambusa balcooa* under cytokinin-based multiplication. However, the minimal polymorphism observed (5.26%) in the stem explant-derived microshoots may reflect explant-related differences, and it is also possible that the more differentiated nature of stem tissue contributes to a higher chance of variation, as suggested by Sharma et al. (2007). This interpretation should be treated cautiously, because ISSR markers on their own cannot fully exclude subtle genomic or epigenetic changes. The three SCoT primers (SCoT 1, 6, and 10) used generated 100% monomorphic bands across all H. crinita samples. However, this complete absence of polymorphism should be interpreted cautiously, because the limited number of primers and bands provides only modest marker coverage. The SCoT results are broadly consistent with studies such as Rathore et al. (2014) on *Cleome gynandra* and Chirumamilla et al. (2021) on *Solanum khasianum*, where no polymorphism was detected, although those studies also varied in primer number, explant source, and regeneration context. By contrast, Bisht et al. (2024), Gautam & Bhattacharya (2021), and Tikendra et al. (2021) reported low levels of variability in *in vitro* regenerated apple rootstock, saffron, and *Dendrobium fimbriatum*, respectively, suggesting that marker outcomes may depend strongly on species, culture conditions, and the extent of marker sampling. Therefore, the SCoT results in the present study support high apparent genetic fidelity, but they may not prove complete genomic conservation.

The comparative analysis of the similarity matrix and dendrogram further clarifies the relationship between the explant source and genetic similarity. The UPGMA clustering revealed that the mother plant (Mo) shared a similarity coefficient of 1.000 with the majority of the regenerants. Notably, the hypocotyl-derived micro shoots (H1–H5) clustered tightly with the mother plant at a coefficient of 1.00, indicating no detectable variation with the marker systems used. The stem-derived microshoots (S2, S3, S4) also clustered closely with the mother plant, suggesting a similarly high level of apparent fidelity. Although regenerants S1 and S5 formed a minor sub-cluster with a slightly lower similarity index (0.977), this deviation should be viewed as a small marker-detected difference rather than evidence of confirmed epigenetic change. While such variation could hypothetically reflect physiological effects (Bairu et al., 2011), culture response or methylation-related processes, the present data do not allow that to be concluded directly. Overall, the clustering pattern suggests that explant differentiation did not have a major detectable effect on marker-based similarity in *H. crinita,* but this interpretation remains constrained by the limited resolution of ISSR and SCoT markers. Also, given that ISSR and SCoT primers are not necessarily species-specific and typically amplify many loci within the genome, the sequencing of multiple amplified loci is often a challenge that limits further validation of sequence similarity in the *H. crinita* samples.

Conclusion

The combination of ISSR and SCoT markers provided a useful comparative assessment of the *in vitro* regenerants, and the agreement between both marker systems indicates high apparent clonal fidelity in *H. crinita* plantlets. However, because ISSR and SCoT markers cover only a limited portion of the genome and the study detected minor polymorphism in some stem-derived regenerants, the results should be interpreted as evidence of high genetic similarity rather than absolute fidelity. Hypocotyl-derived regenerants showed slightly greater stability than stem-derived regenerants in this study, suggesting that explant source may influence the extent of marker-detected variation. Overall, the findings support the use of both explant types for micropropagation, while also providing a molecular baseline for germplasm conservation and future biotechnological improvement of *H. crinita*.

Conflict of interest

The authors declare that they have no competing or conflicting interests.

Acknowledgements

The authors gratefully acknowledge the Laboratory of Plant Cell Technology, Chiba University, Japan for providing support for the *in vitro* regeneration experiments.

Funding information
This work received no internal and external funding

Authors contribution
PNC: Conceptualization, supervision, data analysis, writing, review and editing of manuscript. GMO & AOM: Experimentation, review and editing of manuscript. MSO: Experimentation. DOC: Data analysis, writing and review of manuscript. DYH & PCO: Experimentation.

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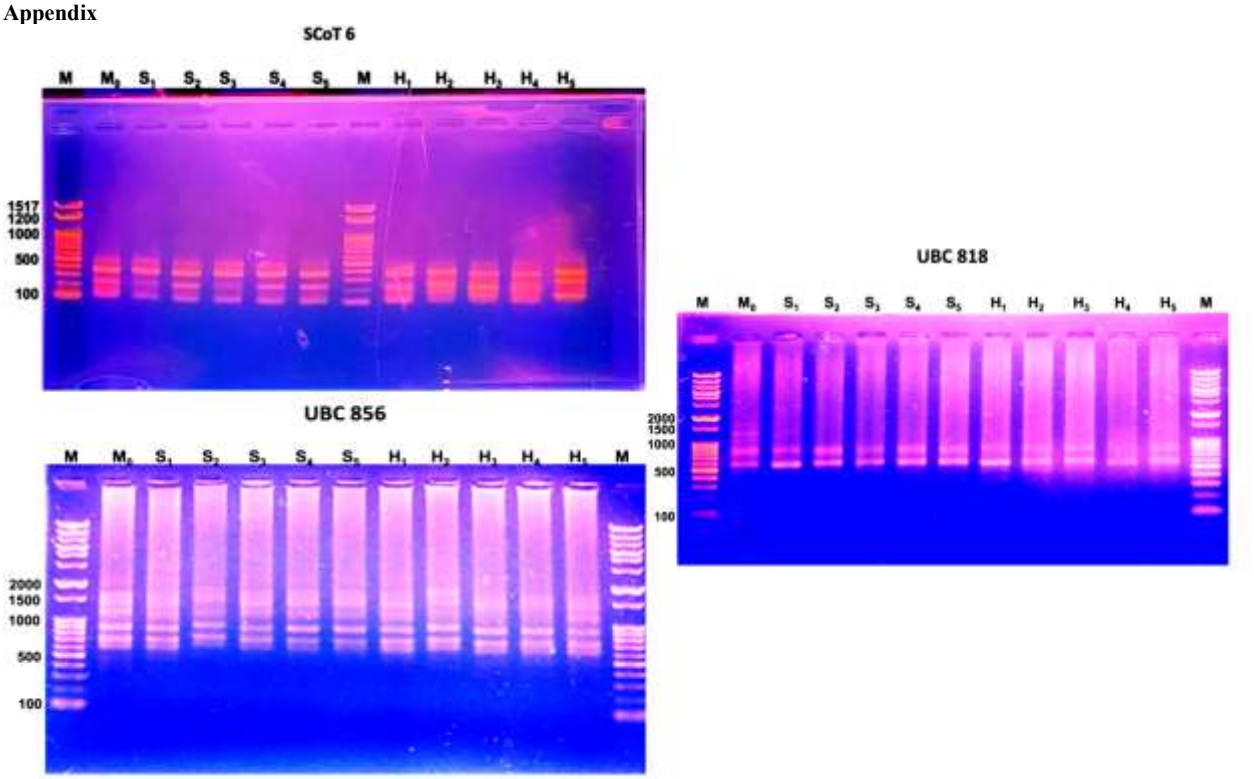

Figure **S1.** Gel images of amplification of ***H. crinita*** regenerants using SCoT6 and ISSR (UBC 818 & 856) primers. M: Gene ladder Wide 2 (0.1 - 20 kbp); Mo: Mother plant; S_1-S_5: shoot-derived regenerants; H_1-H_5: hypocotyl-derived regenerants

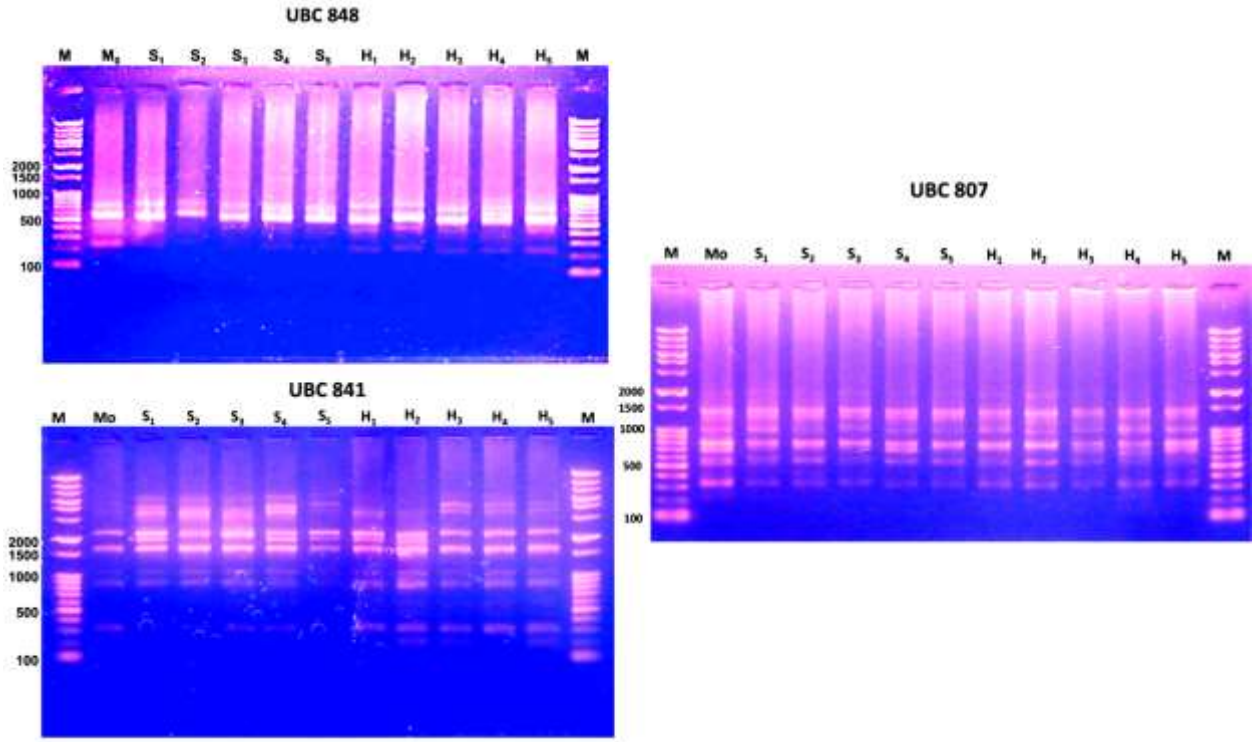

Figure **S2.** Gel images of amplification of ***H. crinita*** regenerants using ISSR (UBC 848, 807 & 841) primers. M: Gene ladder Wide 2 (0.1 - 20 kbp); Mo: Mother plant; S_1-S_5: shoot-derived regenerants; H_1-H_5: hypocotyl-derived regenerants

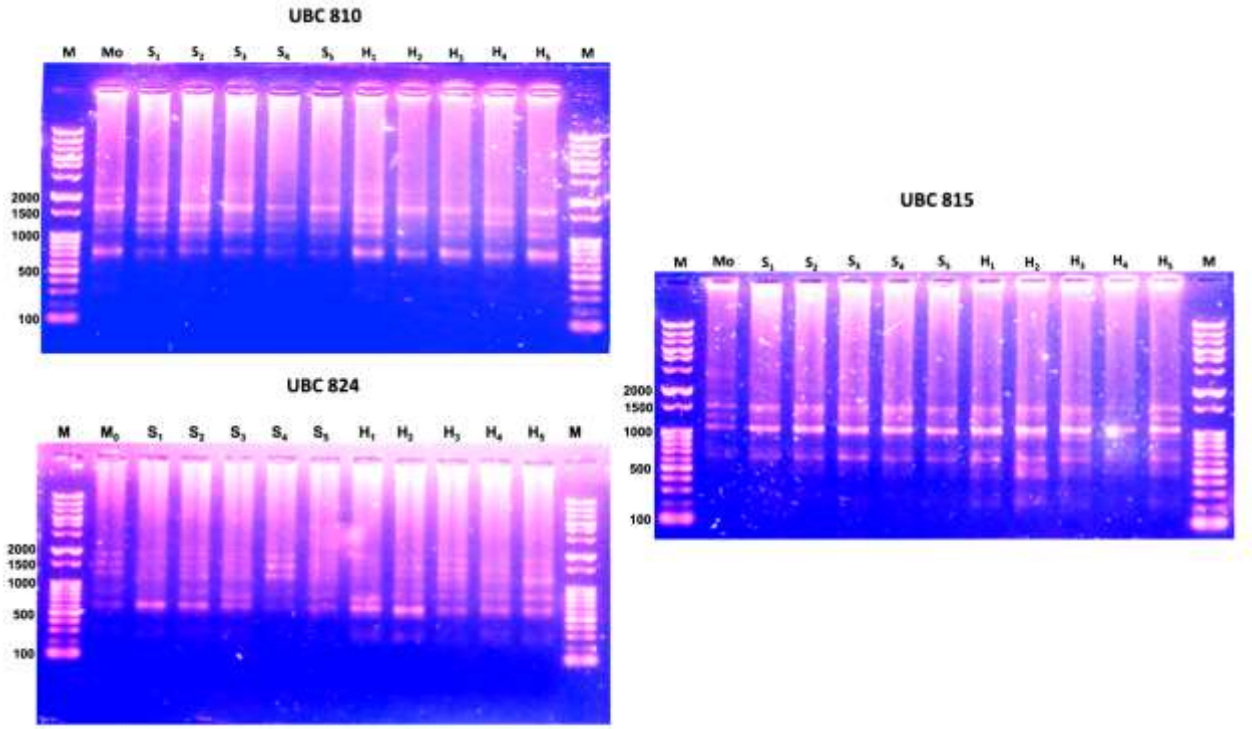

Figure **S3.** Gel images of amplification of ***H***. ***crinita*** regenerants using ISSR (UBC 810, 815 & 824) primers. M: Gene ladder Wide 2 (0.1 - 20 kbp); Mo: Mother plant; S_1-S_5: shoot-derived regenerants; H_1-H_5: hypocotyl-derived regenerants