Journal of Agriculture and Crops
ISSN(e): 2412-6381, ISSN(p): 2413-886X
Vol. 8, Issue. 1, pp: 12-19, 2022
URL: https://arpgweb.com/journal/journal/14
DOI: https://doi.org/10.32861/jac.81.12.19

Academic Research Publishing Group

Original Research **Open Access**

Genetic Stability of *in vitro* Propagated Grapevine *(Vitis vinifera* L.) cv. Al-Bayadi

Bandar S. Aljuaid
Biotechnology Department, College of Science, Taif University, P.O. Box 11099, Taif 21944, Kingdom of Saudi Arabia

Ismail A. Ismail
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Kingdom of Saudi Arabia

Attia O. Attia
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Kingdom of Saudi Arabia

El Dessoky S. Dessoky (Corresponding Author)
Department of Biology, College of Science, Taif University, P.O. Box 11099, Taif 21944, Kingdom of Saudi Arabia
Email: dessoky26@yahoo.com

Article History
Received: 29 October, 2021
Revised: 23 November, 2021
Accepted: 6 December, 2021
Published: 10 December, 2021

Abstract

Grapevine (*Vitis vinifera* L.) fruit crops are a significant source of antioxidants, fibre, and nutrients; all are vital for a healthy diet and play a key role in the economy of several advanced and developing countries. It is of great importance to generate true-to-type plant products using *in vitro* propagation system. Thus, somaclonal variations can multiply very rapidly which leads to loss of the main features of parent rootstocks. In this research, a mixture of three Polymerase Chain Reaction (PCR)-based molecular marker methods – (conserved DNA derived polymorphism) CDDP, (Inter-simple sequence repeat) ISSR, and DNA barcoding – have been used to verify micro propagated grapevine genetic stability. Both ISSR and CDDP primer combinations produced scorable PCR fragments. The total number of bands was 98 and 109, with an average of 9.8 and 10.9 bands/primer in ISSR and CDDP assays, respectively. On the other hand, about 20 polymorphic bands were collected by CDDP primers, of CDDP-3 and CDDP-11produced 1 and 5 bands, with a polymorphism percentage of 11% and 33%, respectively. About 5 different unique PCR bands were detected in the mother plant (control) and were not observed in micro propagated plantlets (MP) samples of grapevine plant or vice versa. The phylogenetic trees were constructed using ISSR and CDDP assays diverged the control from MP samples at 1.3% and 7%, respectively. The phylogenetic tree constructed using (chloroplast *gene* RNA polymerase1) *rpoC1* gene, multiple sequence alignment revealed that *rpoC1* gene sequencing detected small genetic differences between control and MP samples of the grapevine and clustered grapevine control and MP samples to a single cluster with other *Vitis* species. This experiment reveals the potentiality of using CDDP, ISSR, and DNA barcoding in detecting the somaclonal variation of grapevine varieties subjected to tissue culture as a tool for plant conservation and breeding programs.

Keywords: Grapevine *(Vitis vinifera* L.); CDDP; ISSR; DNA-barcoding genes; *RpoC1* gene; Somaclonal variation; Tissue culture.

1. Introduction

Grapevine *(Vitis vinifera* L.) fruit crops are a significant source of fibre, nutrients and antioxidants; all are vital for a healthy diet and play a key role in the economy of several advanced and developing countries. The world's grapevine area totals in 2017 reached 7671879 hectares, producing 91508986 tons, while in Saudi Arabia, area totals reached 3,785 hectares, producing 44,505 tons [1]. Europe accounts for roughly 39% of worldwide manufacturing, preceded by Asia (32%), the Americas (20%), Africa (6%) and Oceania (3%) [2]. Grape domestication resulted in a slight decrease in genetic diversity, suggesting that the grape is a sensible perennial model to study the accumulation of deleterious genetic variability in the lack of a pronounced bottleneck [3].The latter species is cultivated for the manufacturing of fruits of high quality, but it is sensitive to many pathogens. The previous species are mainly used for rootstocks of breeding and fungus-resistant scion cultivars [4].

Traditionally, seeds, cutting, layering, budding, or grafting propagate grapevines, which can be rooted in containers or directly in the soil [5]. Methods of grapevine propagation have been modernized using virus-free breeding stock, mist propagation processes for leafy cutting and fast machine-grafting techniques [6]. Tissue culture is a method to create plant clones from, organs, tissues or single cells *in vitro* under controlled dietary and environmental circumstances. These circumstances include appropriate nutrient supply, medium pH, appropriate temperature and an appropriate gaseous and liquid environment. Resulting clones are similar to the genotype chosen [7].To expand clonal selection could be through somaclonl differences induction, ranging from easy-to-detect deviations in particular morphological features to subtle deviations in strength, bunch, fruit sizes and chemical content [4].

It is of great importance to generate true-to-type plant products using *in vitro* propagation system. Thus, somaclonal variations can multiply very rapidly if induced, which lead to loss the main features of parent rootstocks [8]. In addition, this genetic instability may pose a danger connected with the implementation of *in vitro* cultivation methods for germplasm handling and storage [9].When plant tissue passes through *in vitro* cultivation, several of the

regenerated plantlets no longer appear to be clonal copies of their donor genotype, likely owing to epigenetic modifications. Due to cryptic genetic changes and the growth of somaclones, the wider usefulness of any micropropagation scheme may be restricted [10].

Molecular marker technology is an irreplaceable instrument for genetic research; it utilizes molecular markers of Polymerase Chain Reaction (PCR) to target plant genomic areas. The fragments of PCR could infer genetic variation and population structure of the plant species studied [11]. Furthermore, sophisticated molecular marker analysis may be used to correlate markers of PCR with network of plant genes, giving scientists of plant more control over their genetic resources [12, 13].

Inter-simple sequence repeat(ISSR) screening is intended to target the redundancy of nucleotides situated within plant genomic areas, producing elevated polymorphism and repeatability. It is appropriate for examining genetic variation in multiple plant species. Additionally, it has been successfully utilized for studying somaclonal variation in different plant species, such as grapes [8], rice [14] and bananas [15].

Conserved DNA derived polymorphism (CDDP) is a new technique for producing plant DNA markers for conserved amino acid sequences in proteins. It is suggested that this assay may be used in combination with or as a replacement for other technically simple dominant marker techniques for applications such as targeted quantitative loci mapping [16]. It was used to assess genetic diversity in chickpeas [17] and date palms [18].

DNA barcoding includes sequencing a conventional DNA region as a method for identifying species [19]. A lot of plastid genome areas, *i.e. trnH-psbA*, *rbcL*, *rpoB*, *matK* and *ropC1* have been widely assessed in studying different plant species. The plastid gene *ropC1* has been used to evaluate genetic variation of *Apocynaceae* species [20], *Calluna* [21] and *Gongora* [22].

A mixture of three PCR-based molecular marker methods – CDDP, ISSR and DNA barcoding – has been used in this research to verify the genetic stability of micropropagated grapevine (*V. vinifera* L.) cv. Al-Bayadi.

2. Materials and Methods

Study area: The study was carried out at the labs of Deanship of Scientific Research, Taif University, KSA and Plant Genetic Transformation Department, Agricultural Genetic Engineering Research Institute, Agricultural Research Centre, Giza, Egypt, during the period of March 2017 to June 2019.

2.1. DNA Extraction

The complete genomic of DNA was separated by Alizadeh and Singh's technique [8] from mother plant as control, the six and twelve months micropropagated plantlets (MP) of grapevine plant. DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) was used as directed by the manufacturer, control sample and ten MP grapevine samples were preserved for six and twelve months and were used for DNA extraction, where they were obtained from 5 g tissue. DNA quantity was measured by injecting 2 µl of DNA samples on 1% agarose gel compared to 10 µl of a DNA size marker (Lambda DNA Hind III digest Phi X 174/HaeIII digest) and comparing the degree of DNA sample fluorescence with the various bands in the DNA size marker.

2.2. Primer2s and PCR Analysis

In this research, ten ISSR, ten combinations CDDP primers and *rpoC1* gene region were used (Table 1). The PCR reaction and amplification cycles program were carried out for ISSR, CDDP and *rpoC1* gene according to Collard and Mackill [16], Awad, *et al.* [23] and Phong, *et al.* [24]. The PCR final products were stored at 4^{o}C, PCR fragments were separated using 8% Agarose gel stained with ethidium bromide and documented by Gel Doc XR system (Bio-Rad, Hercules, CA, USA).

Table-1. Forward (F) and Reverse (R) sequences and target for primers of PCR applied in current study.

PN	F	R	Target
ISSR1	AGAGAGAGAGAGAGAGYC	-	(AG)8YC
ISSR2	AGAGAGAGAGAGAGAGYG	-	(AG)8YG
ISSR3	ACACACACACACACACYT	-	(AC)8YT
ISSR4	ACACACACACACACACYG	-	(AC)8YG
ISSR5	GTGTGTGTGTGTGTGTYG	-	(GT)8YG
ISSR6	CGCGATAGATAGATAGAT	-	CGC(GATA)4
ISSR7	GACGATAGATAGATAGATA	-	GAC(GATA)4
ISSR8	AGACAGACAGACAGACGC	-	(AGAC)4GC
ISSR14	CTCCTCCTCCTCCTCTT	-	(CTC)5TT
ISSR15	CTCTCTCTCTCTCTCTRG	-	(CT)8RG
CDDP-3	GCCCTCGTASGTSGT	GCCCTCGTASGTSGT	-
CDDP-4	GCASGTGTGCTCGCC	GCASGTGTGCTCGCC	-
CDDP-5	TGSTGSATGCTCCCG	TGSTGSATGCTCCCG	-
CDDP-6	CCGCTCGTGTGSACG	CCGCTCGTGTGSACG	-
CDDP-7	GGCAAGGGCTGCCGC	GGCAAGGGCTGCCGC	-
CDDP-8	GGCAAGGGCTGCCGG	GGCAAGGGCTGCCGG	-
CDDP-10	GCSGAGATCCGSGACCC	GCSGAGATCCGSGACCC	-
CDDP-11	TGGCTSGGCACSTTCGA	TGGCTSGGCACSTTCGA	-
CDDP-12	AAGGGSAAGCTSCCSAAG	AAGGGSAAGCTSCCSAAG	-
CDDP-16	ATGGGCCGSGGCAAGGTGG	ATGGGCCGSGGCAAGGTGG	-
rpoC1	GTGGATACACTTCTTGATAATGG	TGAGAAAACATAAGTAAACGGGC	-

2.3. Data Analysis

The NCBI BLASTn tool which used to study orthological genes in distinct species [25] was used to download similar sequences for *rpoC1* of the *V. vinifera* gene from a variety of species. PERL scripts were used for editing these sequences [26]. Only one representative gene copy was left for each species. ClustalW tool was used to carry out alignment and phylogenetic analysis of orthological genes [27]. Phylogenetic trees representing gene interactions were built using the ITOL online tool [28].The online tool ClustVis [29] was applied to illustrate the gene diversification assessment outcomes using similarity matrices of sequence alignment. Additionally, the PCR fragments were scored manually using (1) and (0) as present and absent respectively, then it were applied for similarity matrix coefficients between distinct samples for building the phylogenetic dendrogram.

3. Results and Discussion

3.1. ISSR and CDDP Analysis

All ISSR primers used for detecting grapevine somaclonal variation produced scorable PCR fragments (Figure 1). The total number of bands was 98, with an average of 9.8 bands per primer. The ISSR-3 produced the minimum number of bands (6), while primer ISSR15 produced the maximum number of PCR bands (15). The total number of polymorphic bands was 5. All ISSR primers produced monomorphic bands, except for ISSR-8 and ISSR-14, which produced 3 and 2 bands, respectively (Table 2).

Figure-1. The ISSR PCR product of some primers selected for control and ten MP plantlets samples of grapevine

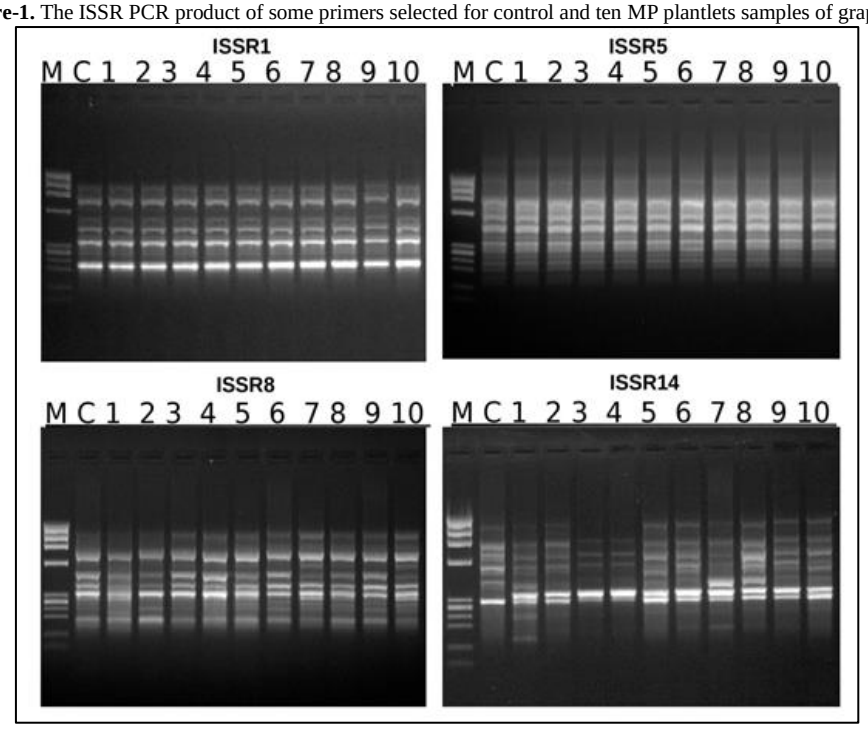
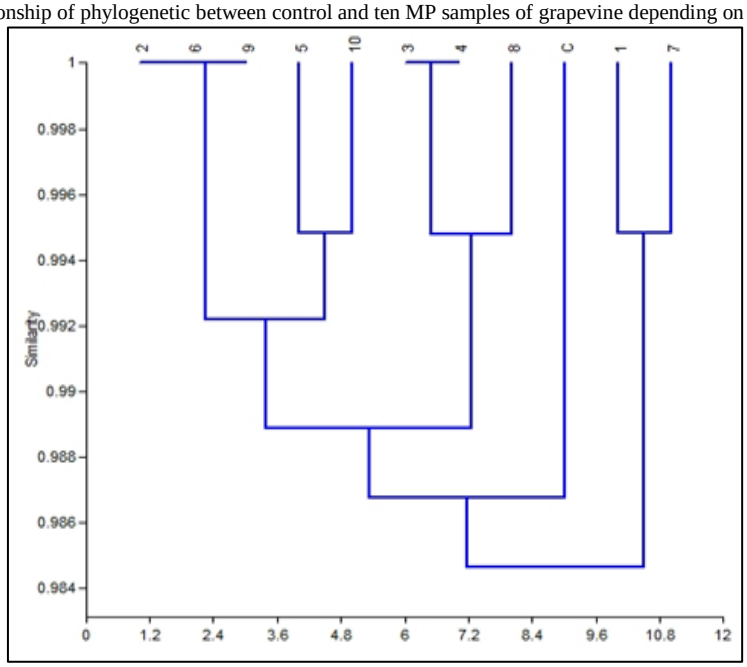


Figure-2. Relationship of phylogenetic between control and ten MP samples of grapevine depending on ISSR binary data



Only ISSR-14 produced a unique band at 330 bp, which was observed in the explant samples and absent in the control. The phylogenetic tree construction based on ISSR binary data revealed that grapevine samples separated at genetic similarity difference of 1.5%. Samples 2, 6 and 9 formed one single cluster as similar as samples 5 and 10, 3 and 4 and 1 and 7. Although control samples were diverged after a genetic difference less than 1.3%, it had more similarity with other grapevine explant samples than samples 1 and 7 (Figure 2).

The ISSR assay was used in long-term micropropagated banana shoots to study genetic stability. The testing produced 47 scorable band classes with 5 ISSR primers, varying in size from 200 bp to 2200 bp. The amount of bands per primer ranged from 7 to 13, with an average of 9.4 bands per primer ISSR, where there were no polymorphic bands [15]. A comparison of ISSR patterns of plantlets acquired after 4 and 6 years of *in vitro* multiplication was performed to check the genetic stability of micropropagated almonds. The testing with the 10 ISSR primers produced 60 scorable band classes, varying from 240 bp to 2.8 kb. For each primer, the amount of bands ranged from 3 to 9, with an average of 6 bands per ISSR [30]. Moreover, micropropagated *Aloe vera* genetic stability was evaluated using ISSR markers [31].Ten ISSR primers generated 73 scorable ISSR bands. For each primer, the number of bands was 4 to 10, with an average of 7.3 bands per primer ISSR. No polymorphism was noted in regenerants obtained after immediate axillary bud regeneration, although RAPD and ISSR reported 80% and 73.3% polymorphism [32].

Table-2. The CDDP and ISSR analysis

PN	TB	MB	PB	PP
CDDP-3	9	8	1	0.11
CDDP-4	12	10	2	0.17
CDDP-5	8	8	0	0
CDDP-6	12	10	2	0.17
CDDP-7	9	7	2	0.22
CDDP-8	14	11	3	0.21
CDDP-10	11	9	2	0.18
CDDP-11	15	10	5	0.33
CDDP-12	9	8	1	0.11
CDDP-16	10	8	2	0.2
ISSR-1	8	8	0	0
ISSR-2	13	13	0	0
ISSR-3	6	6	0	0
ISSR-4	10	10	0	0
ISSR-5	15	15	0	0
ISSR-6	11	11	0	0
ISSR-7	7	7	0	0
ISSR-8	10	7	3	0.3
ISSR-14	8	6	2	0.25
ISSR-15	10	10	0	0

PN: primer name, TB: total number of bands, MB: monomorphic bands, PB: polymprphic bands and PP: percentage of polymorphic bands.

In current study, total numbers of bands 109 were revealed by the CDDP primer combinations, with a mean of 10.9 bands / combination, where scorable PCR fragments were generated by all combinations (Figure 3). Primer combination CDDP-5 produced minimum number of PCR bands (8 bands), while CDDP-11 generated maximum number (15 bands). About 20 polymorphic bands have been collected using CDDP primers, of which CDDP-3 and CDDP-11produced 1and 5 bands, with a percentage of polymorphism 11% and 33%, respectively (Table 2). According to CDDP assay, 6 different unique bands which are present were retrieved in the control and were absent in different grapevine MP samples. These PCR fragments have been produced using primers CDDP-8 (305 and 230 bp), CDDP-10 (10 bp), CDDP-11 (1860 and 1480 bp), and CDDP-16 (1460 bp).

CDDP assay was used to characterize the genetic diversity in chickpeas, ten primers produced 151 bands, 141 bands of which were polymorphic, with 14.1 as an average bands per primers ranging from 9 to 17 [17].

Figure-3. The CDDP PCR product of some primer combinations selected for control and ten MP samples of grapevine

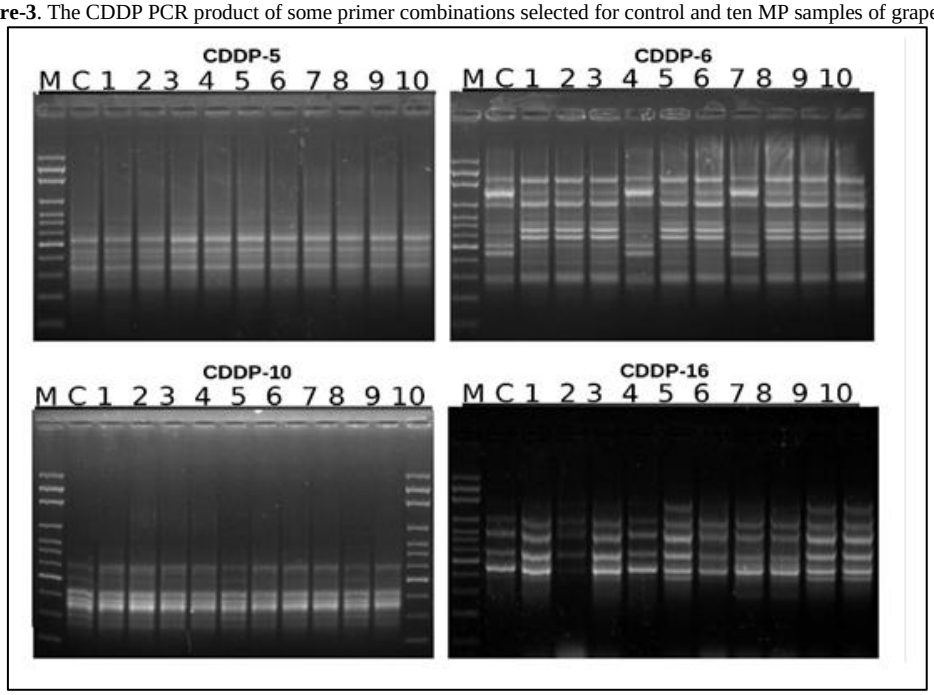
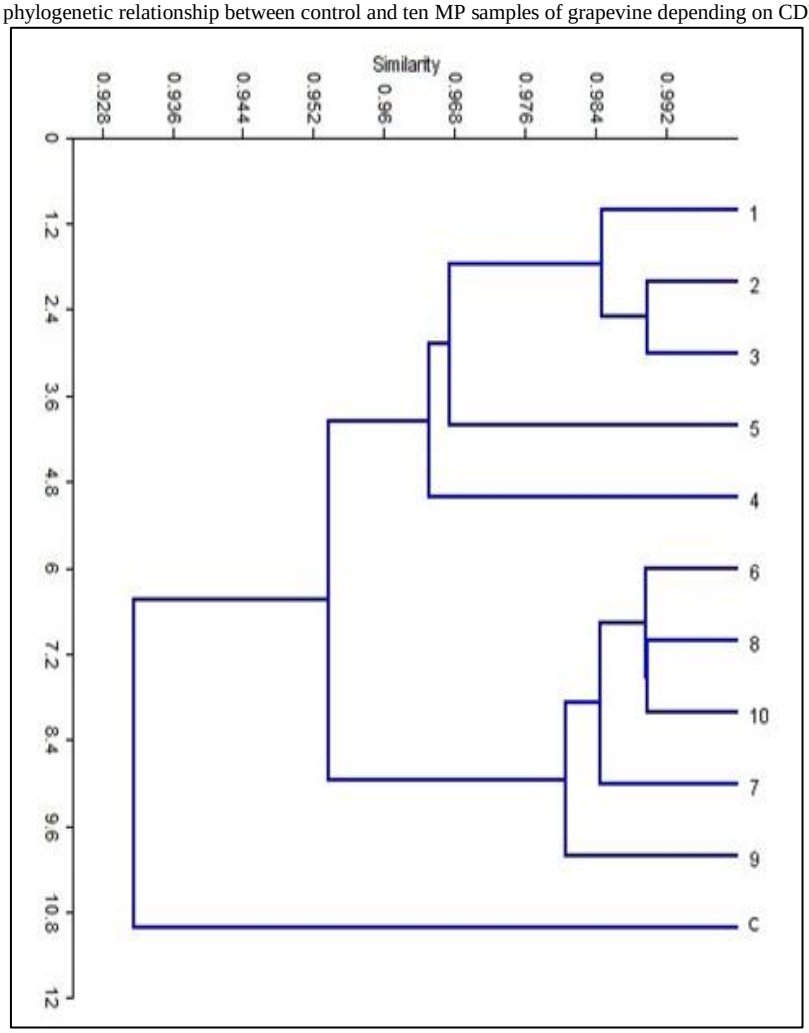


Figure-4. The phylogenetic relationship between control and ten MP samples of grapevine depending on CDDP binary data



Genetic variation evaluation of bittersweet germplasm utilizing CDDP disclosed limited genetic diversity among distinct populations and demonstrated CDDP's potential with further investigation and collection of samples across a wider worldwide distribution spectrum for genetic improvement of Solanaceae plants [33].

In current study, Phylogenetic divergence depending on CDDP data revealed that the different grapevine explants and control samples were genetically differentiated at 7% similarity difference. Samples such as 6, 8, 10, 7 and 9 were clustered in one group at a similarity 97.6%, while samples 1, 2 and 3 were clustered at 98.4% similarity. On the other hand, samples 5 and 4 of MP were expelled from other samples at 3.5% of genetic dissimilarity.

Moreover, the control observed a genetic divergence at a similarity score of 93% with a dissimilarity of 7% with all of the grapevine MP samples (Figure 4).

In order to strengthen the genetic diversity analysis power of distinction, both ISSR and CDDP binary have been combined for phylogenetic tree construction. The phylogenetic analysis differentiates between different grapevine samples at 0.03% genetic dissimilarity. The control sample has a dissimilarity score of 6% with all grapevine MP samples. The phylogenetic tree was then diverged into two groups – samples 9, 6, 10, 7 and 8 in one group and samples 3, 1, 2, 4 and 5 in another (Figure 5). The similarity matrix heat map using CDDP and ISSR data illustrated that the control sample is highly diverged from most of the grapevine explant, except for samples 7, 4 and 3 which have a low genetic difference compared with other MP and in comparison with the control sample (Figure 6).

Figure-5. The phylogenetic tree of control and ten MP samples of grapevine using both ISSR and CDDP binary data

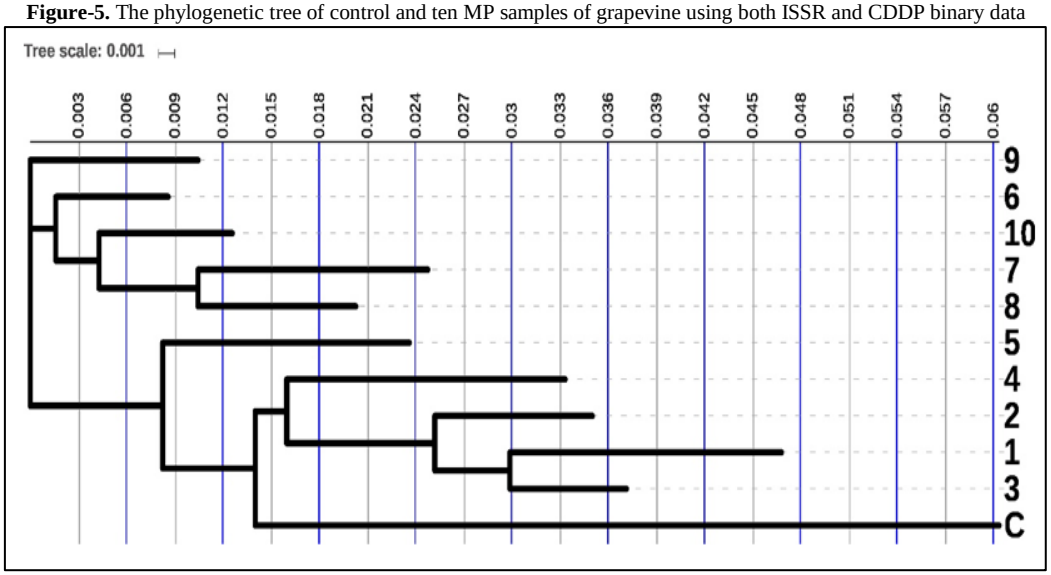
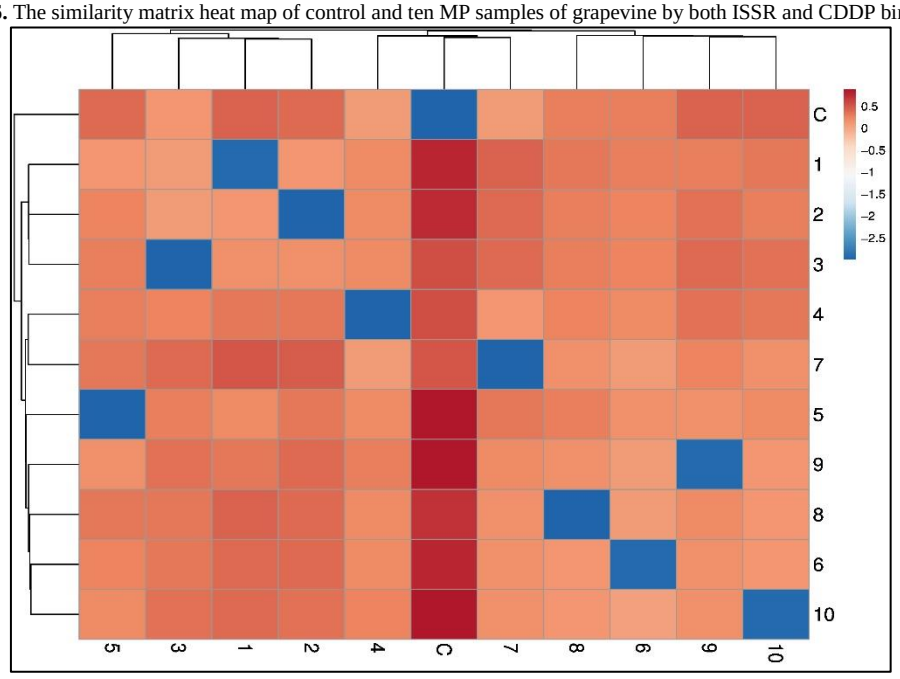


Figure-6. The similarity matrix heat map of control and ten MP samples of grapevine by both ISSR and CDDP binary data

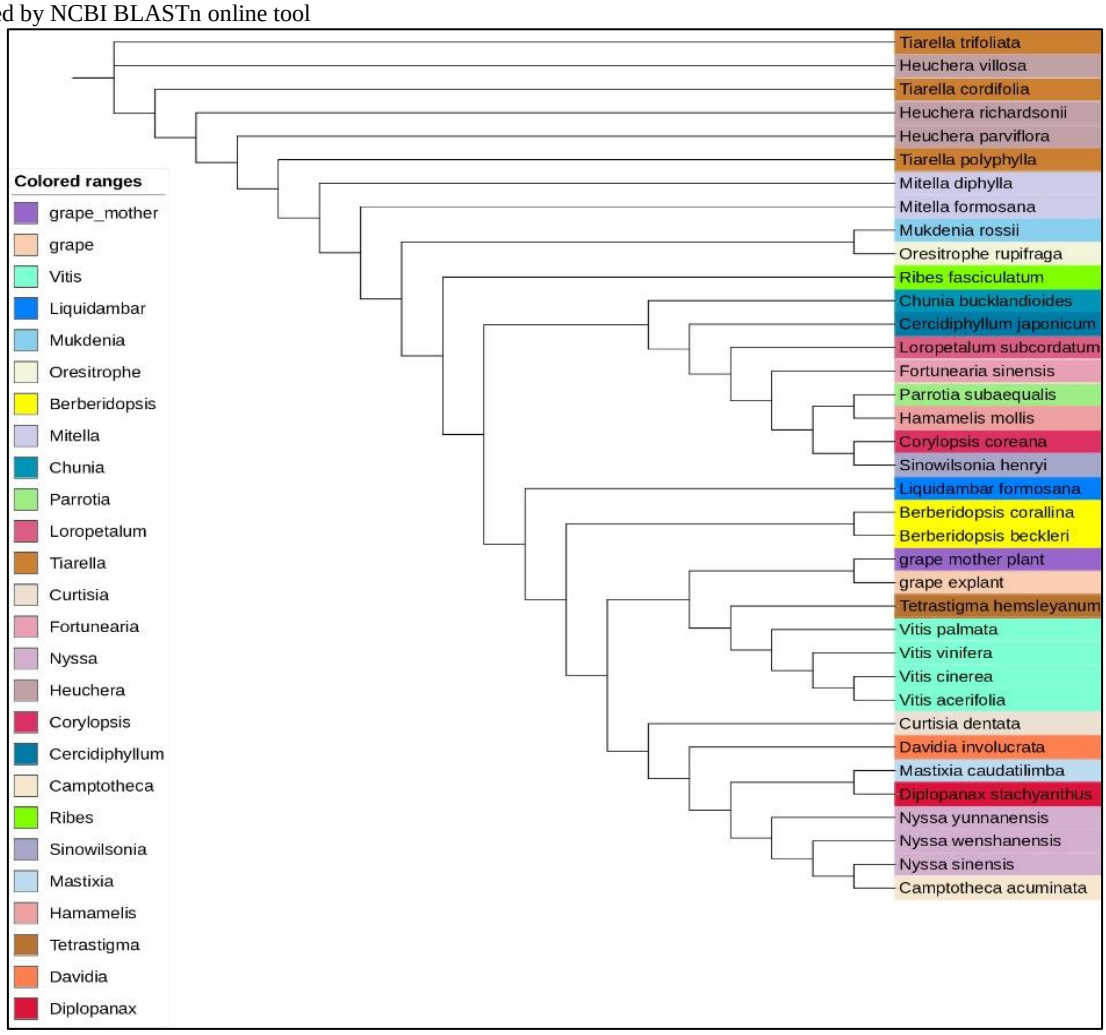


3.2. DNA Barcoding of Somaclonal Variation Using *rpoC1*

The chloroplast *rpoC1* gene was applied to infer the genetic similarity and difference resulted by grapevine tissue culture propagation. Both the control and one explant tissue sample were sequenced for the *rpoC1* gene. The generated DNA sequences were used to measure the possible effect of the tissue culture propagation on grapevine diversity. The grapevine *rpoC1* gene of both the control and MP were compared to 35 plant species retrieved by the NCBI BLASTn online tool. The phylogenetic tree constructed using *rpoC1* genes multiple sequence alignment revealed that sequencing of *rpoC1* gene detected small genetic differences among the control and MP samples of grapevines. This could be due to the mutation effect of the tissue culture propagation procedure on chloroplast genes or the low rate of *rpoC1* mutation in comparison with other genes. Successfully, the *rpoC1* assigned both grape samples (control and MP) to a single cluster, where different *Vitis* species *i.e. V. palmata*, *V. vinifera*, *V. cinerea* and *V. acerifolia* have been grouped. This could infer the usefulness of these genes in the identification of wild and cultivated grape species. In another context, *rpoC1* failed to differentiate between *Tetrastigma* species and other

plant species, which could infer its weakness in the identification of this species (Figure 7).

Figure-7. The phylogenetic tree analysis comparing grapevine *rpoC1* gene of control and ten MP samples with 35 plant species retrieved by NCBI BLASTn online tool



4. Conclusion

It is extremely important to detect somaclonal variation in tissue culture programs, and delivering fast and cost-effective tools is essential. The ISSR and DNA barcoding marker assays have a low ability to detect genetic variation caused by *in vitro* propagation of grapevines. This could be related to the minor effect of propagation on areas targeted by such markers. On the other hand, CDDP marker assays have successfully detected higher numbers of genetic variation between control and MP samples. This could encourage both researchers and grapevine breeders to integrate these particular assays in the propagation system, in order to identify true-to-type plants and select highly mutated plants for conservation and breeding programs.

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