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Assessment of genetic diversity of grapevine (*Vitis vinifera* L.) cultivars grown in Pakistan using ISSR markers

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Abstract
Many American and European cultivars of grapes are grown in several countries. However, their interrelationship is indistinct. Therefore, ampelographic and genetic characterizations were conducted to analyze and identify the similarities and relatedness among seven different grapevine cultivars, namely Red Globe, Autumn Royal, Crimson Seedless, Thompson, Perlette, King Ruby, and Sundar Khani (from Pakistan). Morphological characteristics examined include berry morphology, fruit skin color, flesh color, sweetness, compactness, and weight of grape bunch. Molecular diversity of the cultivars was evaluated using the Inter-Simple Sequence Repeat (ISSR) markers. The results showed that morphological characterization differed among the grapevine cultivars. Six primers examined yielded 84 scored bands ranging from 150 bp to 1200 bp. The polymorphism information content (PIC) values ranged from 0.233 to 0.457, while the amount of polymorphism varied with each primer, from 83% to 100%. Our results showed that the fingerprints of ISSR markers are a proficient method for identification and resolution of genetic diversity among different grape cultivars.

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Introduction

Grapevine (*Vitis* spp.) is a valuable economic resource that is widely grown around the world. The grapevine has become one of the most significant plants in the world due to its numerous applications,

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particularly in wine, food, and beverages. It also has beneficial health effects as the phytochemical composition of grapevine includes a great variety of flavonoids that have been shown to exhibit varied health benefits, including anti-cancer, antioxidant, and cardiovascular disease prevention, as well as the treatment of several skin conditions (Ali et al., 2010; Pezzuto et al., 2022).

The study of genetic relationship and molecular diversity of various cultivars of grapes is used to determine their evolutionary perspectives. Genetic study is also helpful for breeding and preservation of the germplasm of plant cultivars (Chadha and Randhawa, 1974; Salgotra and Chauhan, 2023). However, vegetatively propagated plant cultivars generate difficulties for their proper and accurate identification.

Molecular markers have an imperative role in the identification and characterization of genetically different plant cultivars. These markers have been evidenced as powerful tools in providing information on polymorphism at the DNA sequence level, and their use for grapevine identification is regarded as an alternative or easy ampelography (Akhare et al., 2008; Bahurupe et al., 2013; Barrias et al., 2023). ISSR (Inter-Simple Sequence Repeat) markers are regarded as particularly informative in genetic diversity investigations (Bornet and Branchard, 2001; Assefa et al., 2023). The use of ISSR markers is a fast, simple, cost-effective, highly stable, and PCR-based technique, which needs no prior information on the sequence, and provides a fast marker system for many organisms, including plants (Modgil et al., 2005; Kandasamy et al., 2013; Assefa et al., 2023; Salimov et al., 2024). The use of ISSR markers is based on inter-tandem repeats of short DNA sequences found inside microsatellite repeats, which are valuable in measuring genomic diversity since they indicate variation at multiple loci at the same time (Zietkiewicz et al. 1994; Mint Abdelaziz et al., 2020). This method gives information on the characterization of genetic similarities across populations, fingerprinting or genetic diversity analysis, detection of clonal deviations, cultivar identification, phylogenetic analysis, revealing of genomic variability, and hybridization evaluation (Hassan et al., 2011; Joshi et al., 2013; Salimov et al., 2024).

The current study was aimed at using morphological characteristics, such as berry/fruit skin color, flesh color, sweetness, compactness and weight of grape bunch, and molecular characterization using ISSR markers, to analyze the intensities of genetic variability amongst the selected seven potential grape cultivars. To our knowledge, this is the first description of ampelographic characterization showing morphological and genetic relationships among these cultivars.

Materials and Methods

Plant material

Seven cultivars of *Vitis vinifera* (Red Globe, Autumn Royal, Crimson Seedless, Thompson, King Ruby, Perlette, and Sundar Khani) were bought from different nurseries in Pakistan (**Table 1**). Cultivars Red Globe, Autumn Royal, Thompson, Perlette and King Ruby originated from the United States of America, cv. Crimson Seedless originated from Italy, and cv. Sunder Khani from Pakistan. Cultivars Sundar Khani and King Ruby were bought from the Ali Nursery, Kasur. Cultivar Red globe was bought from Model Town Nursery, Lahore. Cultivars Thompson, Autumn Royal and Crimson Seedless were bought from Peshawar. All cultivars were planted in the Botanical Garden, University of the Punjab, Lahore (N 31° 30' 4.3236", E 74° 18' 5.4684"). Soil was first ploughed then amended with compost/manure and fertilizer at a rate of 100 g m^{-2} to increase the soil organic matter and inorganic nutrient contents. Inter-row spacing of the grape plants was 120 to 180 cm, and the rows were spaced at 300 cm distance to achieve best airflow and maximum sunlight**.**

Plants were fertilized twice a month with a general-purpose fertilizer containing equal amounts of nitrogen, phosphorus, and potassium (N-P-K). Grapevines were trained using double Kniffin system by installing fence bearing the weight of vigorously growing plants. Data collected for morphological characteristics include, seed characters, fruit skin color, flesh color, bunch compactness and fruit shape. These characteristics were recorded by visual check. Sweetness was confirmed by taste. Weight per bunch was measured using a digital balance.

Table 1. Origin and characteristics of grapevine cultivars from different areas of Pakistan

Cultivar	Consumption	Seeded/Seedless	Origin	Collection Area
Red Globe	Table grapes	Seeded	California, USA	Peshawar
Autumn Royal	Table grapes	Seedless	California, USA	Peshawar
Crimson Seedless	Table grapes	Seedless	Italy	Peshawar
Thompson	Table Grapes/Wine/Dried Raisins	Seedless	California, USA	Lahore
Sunder Khani	Table grapes/Dried raisins	Seedless	Peshawar, Pakistan	Lahore
Perlette	Table grapes	Seedless	California, USA	Kasur
King Ruby	Table grapes	Seedless	USA	Kasur

Isolation of plant genomic DNA

The total genomic DNA was extracted from fresh and young leaves of the seven grapes cultivars using the FavorPrep Plant Genomic Extraction kit cat No. FAPGK001-1 according to the manufacturer's instructions.

ISSR analysis

Six primers ((GGAT)4, (CAA)5, (GACA)4, (CA)8, (GATA)4, and (GTG)5) were employed for the ISSR analysis. These primers were provided by the Microbiology & Molecular Genetics Department of the University of Punjab, Lahore. Each amplification mixture of 25 µL contained: 2 µL DNA sample (30-40 ng), 3 µL of each primer (10 pmoL), 0.5 µL Taq polymerase (5 units/µL), 12.5 µL of Mater Mix (PCR Buffer (10X), dNTPs (20 mM), $MgCl_2$ (25 mM) and 7.0 µL ampoule water. The amplification reaction was carried out on a Convergys TC96 thermal cycler. The first cycle included one minute of denaturation at 94 °C, followed by 35 cycles of 20 seconds at 92 °C. The primers were annealed for 1 minute at the prescribed temperature for that primer, followed by primer extension at 72 °C for 20 seconds, and a final extension at 72 °C for 2 minutes.

The amplified products were electrophoresed in 1.7% agarose gel with ethidium bromide 0.5 µg mL^{-1} in 1X TBE (0.089 M tris base, 0.089 M boric acid, and 0.002 M EDTA) for 3 h at 100 V. Before electrophoresis, each primer received 2.5 µL of the loading buffer (10× TAE, 50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol).

After electrophoresis, the gels were visualized in a UV transilluminator and photographed with the Gel Documentation System (UVItec, Cambridge, UK).

Cluster analysis

Clear, distinct, and reproducible bands were selected for checking the molecular diversity. Genetic distance between the seven cultivars of grapes was calculated using the Jaccard Coefficient (1901) method. For the cluster analysis, UPGMA (Unweighted Pair Group Method using arithmetic means) was applied. The UPGMA generated similarity matrix was used to construct a dendrogram using the PAST 4.03 software to detect cluster variation among different cultivars of grapevine using different primers. Similarity and genetic distance indices were also calculated.

Results

Ampelographic characteristics of grapevine cultivars

In describing the morphological characteristics, only cv. Red Globe was seeded, while the other cultivars were seedless. The color of berries varied among the cultivars, where it was purple in Autumn Royal, deep red in King Ruby and Crimson Seedless, rosy red in Red Globe, and green in Thompson, Sunder Khani and Perlette. Whitish flesh color was observed in King Ruby, Perlette and Thompson, bright yellow color in Autumn Royal and Red Globe, and green flesh was observed in Crimson Seedless and Sunder Khani. Berries were round in Perlette and Red Globe, oval in King Ruby, Thompson and Autumn Royal, cylindrical in Crimson Seedless and obligate in Sunder Khani. Phenoleptic property (i.e., taste) of Sunder Khani, Autumn Royal and King Ruby fruits indicates that they are very sweet, compared to Red Globe, Perlette and Thompson being less sweet, and Crimson Seedless fruit was sweet with tartness (**Table 2**).

Table 2. Berry morphology of grapevine cultivars

Cultivar/ Parameter	Red Globe	Autumn Royal	Crimson Seedless	Thompson	Sunder Khani	Perlette	King Ruby
Skin color	Rosy red	Purple	Deep red	Green	Green	Green	Deep red
Flesh color	Bright yellow	Bright yellow	Green	whitish	Green	Whitish	Whitish
Fruit shape	Round	Round to Oval	Cylindrical	Oval	Obligate	Round	Round
Fruit							

* High-quality images of grapevine cultivars were taken from various links on Google Images.

Maximum mean bunch weight was observed in Sunder Khani (1,230 g) followed by King Ruby (824 g), Thompson (816 g), Crimson Seedless (766 g), Perlette (651 g) and Red Globe (503 g), while the lowest bunch weight was recorded in Autumn Royal (373 g). Grapes of cv. Thompson were loosely arranged, while those of King Ruby were semi-compact and compact in the other cultivars (**Table 3**).

Table 3. Phenoleptic and bunch characteristics of grapevine cultivars

Cultivar	Taste	Mean wt. (g)	Bunch compactness
Red Globe	Less sweet	503	Compact
Autumn Royal	Very sweet	373	Compact
Crimson Seedless	Sweet with tartness	766	Compact
Thompson	Sweet	816	Loosely arranged
Sunder Khani	Very sweet	1230	Compact
Perlette	Less sweet	651	Compact
King Ruby	Very sweet	824	Semi-compact

ISRR marker-based characterization

Five of the six ISSR primers employed in the study were determined to be polymorphic, and **Table 4** shows the results obtained utilizing these primers. Data from the six ISSR primers yielded 17 DNA fragments, 16 of which were polymorphic and one monomorphic. The number of bands varied from 1 to 6 for each primer, with an average of 2 bands per template (**Figure 1a-e**).

Table 4. Data of polymorphic ISSR primers used

Primer sequence (5'→3')	TN	Tm (°C)	PB	MB	TB	%P	PIC	AS (bp)
(GTG)5	15	59.4	3	0	3	100	0.38	600 to 900
(GACA)4	16	53.3	3	0	3	100	0.306	600 to 1200
(CAA)5	15	45.0	3	0	3	100	0.299	700 to 1200
(GATA)4	16	36.2	5	1	6	83	0.233	150 to 500
(CA)8	16	55.1	2	0	2	100	0.457	200 to 500
Total			16	1	17	-	-	-
Mean			3.4	0.2	3.2	96.6%	0.335	-
Range			-	-	-	83-100	0.457	150 to 1200

TN, number of nucleotides; Tm, melting temperature; PB, number of polymorphic bands; MB, number of monomorphic bands; TB, number of total bands; %P, percent polymorphism; AS, amplicon size; bp, base pairs.

PCR-amplified DNA fragments ranged from 150 to 1200 bp. Primer $(GATA)_4$ had the most amplified bands (6) (**Figure 1d**), while primer $(CA)_8$ had the fewest (2) (**Figure 1e**). Primers $(GACA)_4$, $(CA)_8$, $(GTG)_5$, and $(CAA)_5$ displayed the maximum polymorphism (100%), while the lowest polymorphism (83%) was obtained with primer (GATA)4 (**Figure1 a-e**). The average percent polymorphism among the grapevine cultivars detected by the ISSR primers in the present investigation was 96%. No amplification was observed with primer (GTG)5 in cultivars Crimson Seedless and King Ruby, while in the other cultivars (Thompson, Autumn Royal, Red Globe, Perlette and Sundar Khani) fragments were found to be in the range of 600 to 900 bp (**Figure 1 a**). With primer (GACA)4, King Ruby showed no amplification, while the amplification pattern of the other grape cultivars ranged from 700 bp to 1200 bp (**Figure 1 c**).

The DNA amplification pattern of the grape cultivars with primer (CAA)5 ranged from 700 to 1000 bp (**Figure 1 c**). The cultivar King Ruby did not produce any bands with this primer. In the case of primer (GATA)4, all the cultivars showed bands ranging from 200 to 500 bp. The bands produced with primer (CA)8 were in the range of 300 to 500 bp (**Figure1 e**).

Of the six primers used, (GGAT)4 did not show any banding pattern with any of the cultivars studied as this primer was non-transferable and may have null alleles for which it did not find where to bind for amplification. All of the primers were polymorphic, with a PIC (Polymorphism Information Content) of < 0.5. As a result, these polymorphic primers can be used to accurately measure genetic diversity in grapevine varieties. The average PIC value for all ISSR primers was 0.335, indicating that all of the markers employed in this investigation were dominant.

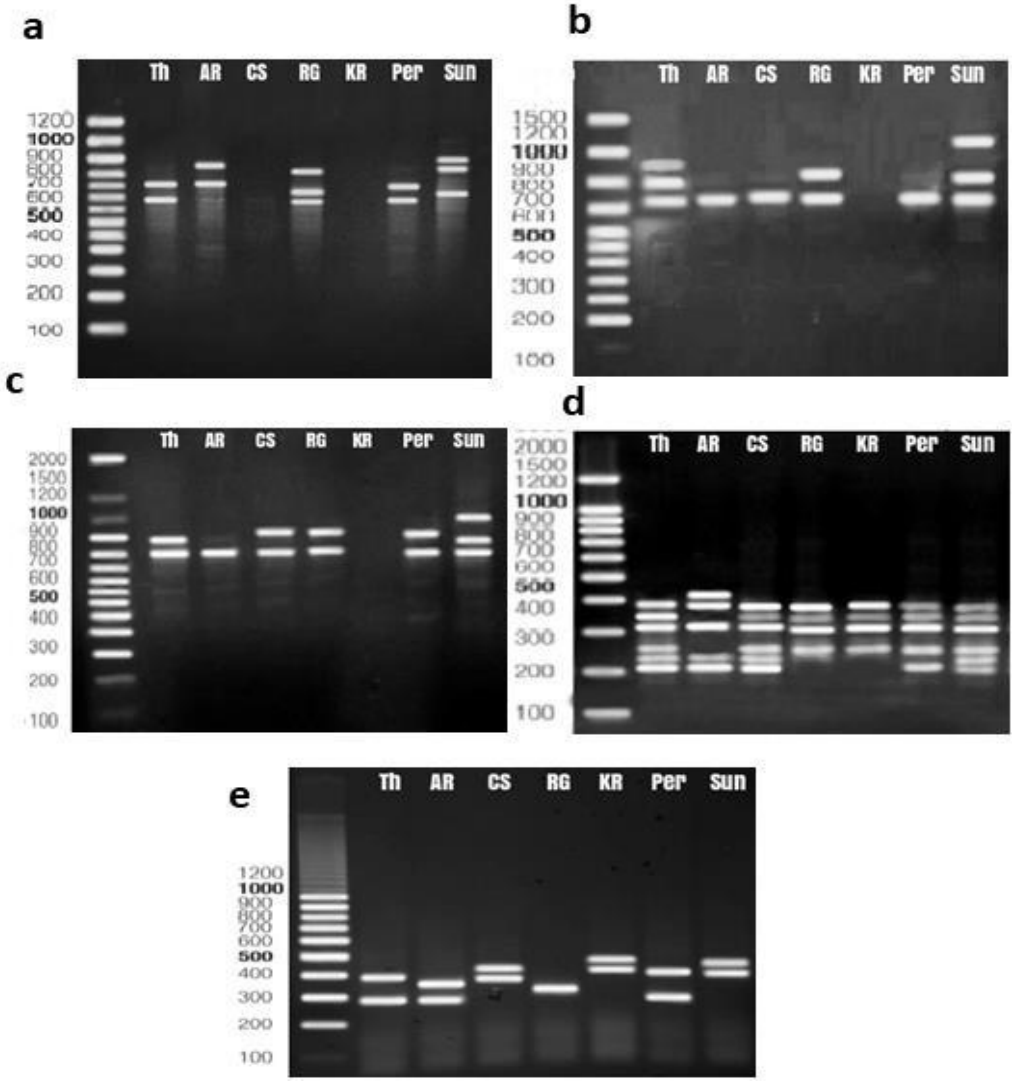

Figure 1. PCR amplified product showing different banding pattern in grape cultivars with primers (a) (GTG)5, (b) (GACA)4, (c) $(CAA)_5$, (d) (GATA)4 and (e) (CA)8. Th, Thompson; AR, Autumn Royal; CS, Crimson Seedless; RG, Red Globe; KR, King Ruby; Per, Perlette; Sun, Sunder Khani.

Formation of a dendrogram

Jaccard's coefficients were used to compare a set of variables and create a similarity matrix. The Jaccard coefficient values for all genotypes are provided in **Table 5**. Similarity indices were determined using 6 ISSR primers, ranging from 12% (between Sundar Khani and Thompson) to 79% (Red Globe and Crimson Seedless), suggesting a considerable genetic diversity among the grapevine cultivars examined here. **Figure 2** depicts the dendrogram (Jaccard's distance, paired group) clustered with the data obtained by all primers and their amplicons, which grouped the seven grapevine cultivars into two primary clusters, namely cluster A and cluster B that displayed 12% similarity cutoff value. Cluster A included only one cultivar, Sundar Khani, while the other six cultivars are located in Cluster B. Cluster B was further separated into sub-clusters B1 and B2, with 31% similarity. The sub-cluster B1 has five cultivars and was separated into sub-clusters B1:1 and B1:2 that

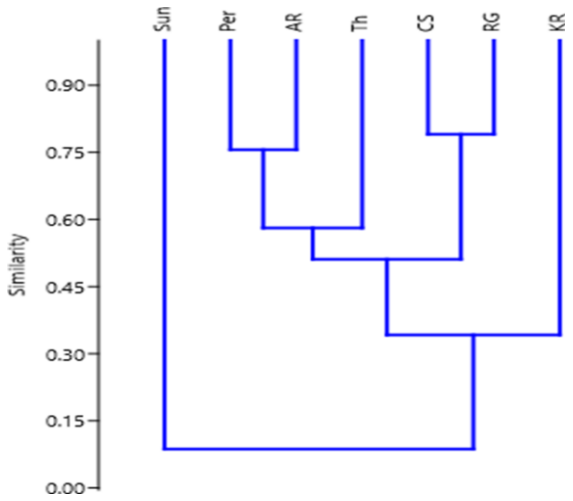

Figure 2. An UPGMA cluster dendrogram showing the genetic relationships among seven grapevine cultivars based on six ISSR markers. Th, Thompson; AR, Autumn Royal; CS, Crimson Seedless; RG, Red Globe; KR, King Ruby; Per, Perlette; Sun, Sunder Khani

shared 47% commonalities. The sub-clusters B1:1 and B1:2 have three (Perlette, Autumn Royal, and Thompson) and two (Crimson Seedless and Red Globe) cultivars, respectively. Sub-cluster B1:1 was further separated into two clusters, one with Perlette and Autumn Royal and the other with Thompson. The sub-cluster B2 contained only one cultivar, King Ruby. Cultivars Crimson Seedless and Red Globe had the maximum closeness (79%), followed by Perlette and Autumn Royal at 75%. Cultivars Sundar Khani and King Ruby were found to be the most varied cultivars.

Table 5. Similarity and genetic distance indices among seven grapevine cultivars obtained from the ISSR marker analysis

Ecotype	Th	AR	CS	RG	KR	Per	Sun
Th	1	0.6614	0.5	0.3952	0.1889	0.5	0.1889
AR	0.3386	1	0.7559	0.5976	0.2857	0.7559	0.1889
CS	0.5	0.2441	1	0.7905	0.3779	0.5	0.5
RG	0.6048	0.4024	0.2095	1	0.478	0.3162	0.3952
KR	0.8111	0.7143	0.6221	0.522	1	0.3779	0.125
Per	0.5	0.2441	0.5	0.6388	0.6221	1	0.25
Sun	0.875	0.8111	0.5	0.6048	0.875	0.75	1

Th, Thompson; AR, Autumn Royal; CS, Crimson Seedless; RG, Red Globe; KR, King Ruby; Per, Perlette; Sun, Sunder Khani. Similarity (Above); Genetic distance (Below).

Discussion

This study employed the use of morphological and molecular characterizations to comprehensively identify seven different grapevines cultivars. Morphological characterization showed that all cultivars were different from each other. Knezovic et al. (2017) studied morphological and genetic characteristics of grape cultivars in Herzegovina and found a broad value range of similarity coefficient for both methods. Similar findings were earlier reported by Nieddu et al. (2007) using Sardinian, Bovale and Spanish grape cultivars. In another study, Spanish cultivars of *Vitis vinifera* were characterized comprehensively using morphological characters and microsatellites in Madrid and found the cultivars to be distinctly different from each other (Ortiz et al., 2004).

The fingerprinting technique is useful for analyzing varietal or genetic diversity, determining genetic similarity between populations, revealing clonal dissimilarity, identifying cultivars, conducting phylogenetic studies, assessing genomic instability, and evaluating hybridization (Bidyananda et al., 2024). Because of their unique identification, varieties and cultivars have shown high genetic variability and divergence both within and between them. Genetic variation gives information about the gene pool for future generations while also preventing the loss of genetic resources used in breeding operations (Salgotra and Chauhan, 2023).

ISSR (Inter-Simple Sequence Repeats) markers are co-dominant markers. They need no prior sequence and are easy, rapid and consistent. They can distinguish heterozygotes from homozygotes (Moreno et al., 1998; Gemmill and Grierson, 2021). Moreover, ISSR markers are more appropriate for the evaluation of the genetic diversity as these markers are very efficient in revealing polymorphism in very closely related germplasms as the minimal differentiation among the samples can be observed (Alansi et al., 2016). Besides cultivar identification, ISSR markers can be used to describe intra-varietal variability in grapevines while also identifying cultivars (Dhanorkar et al. 2005; Salgotra and Chauhan, 2023). ISSR markers have been used to analyze a variety of grapes (Kandasamy et al., 2013). ISSR markers (UBC 810,811, 815, 834, and 850) have been shown to be more efficient in grapevine cultivar identification because of their high polymorphism, repetitive motifs and lower cost, compared to AFLP, RFLP, and RAPD (Malik et al., 2014; Chen et al., 2020). ISSR markers are considered to be the best in evaluating the genetic variability and polymorphism of grape cultivars (Choudhary et al., 2014; Salimov et al., 2024). Even a small set of ISSR primers can be used to discriminate among grape cultivars (Prins et al., 2009).

In this study, all the primers used resulted in polymorphic bands ranging from 36.13% to 100%, although cv. Crimson Seedless showed no banding pattern with (GTG)5, and King Ruby with primers (GTG)5, (GACA)4 and (CAA)5. Variations in the number of bands from the primers may be due to differences among the grape cultivars and the concentration of the agarose gel (Powell et al., 1996; Amein et al., 2020).

The ISSR data was applied to evaluate genetic association among 16 table grape varieties (*Vitis* spp.) from Turkey (Sabir et al., 2008). Among 50 primers, they noted that 14 primers generated a total of 110 clear DNA fragments including 88 polymorphic (80.5%) fragments. Previously, Herrera et al. (2002) used ISSR markers to compare among four grape cultivars in Chile. Also, Argade et al. (2009) reported ISSR analysis applied on grapevine cultivars. Dhanorkar et al. (2005) found a 96%

polymorphism rate in a genetic assessment study of *V. vinifera*, *V. labrusca*, and *V. rotundifolia* cultivars using 13 ISSR primers. The ISSR markers utilized in the current study revealed a significant polymorphism rate between Thompson, Autumn Royal, and Sunder Khani. This further supports previous findings that the use of ISSR primers could be an effective and trustworthy technique for identifying grape varieties (Sabir et al., 2008; Hbyaj et al., 2024).

However, despite ISSR markers being beneficial for identifying plant cultivars, some primers produced unsatisfactory results. For example, primer (GGAT)4 yielded no results for any of the grapevine cultivars tested in this study. As noted by Ng and Tan (2015), one of the main challenges of ISSR analysis relates to a lack of reproducibility due to spurious bands. Therefore, to find an optimal primer, it is necessary to first test for high polymorphism, repeatability, and resolving power. After the screening for appropriate primers, the selected primers may yet vary in effectiveness, resulting in some errors.

Conclusion

This study could be used as a preliminary investigation to determine the likelihood of an allelic hierarchy forming as an internal control for DNA fingerprinting of grape varieties. Additional genetic research should be conducted using more cultivars, primers, and marker systems. Cultivars with high genetic diversity should be properly conserved; nevertheless, cultivars with low genetic diversity should also be conserved because they may have certain unique qualities that can be valuable in crop improvement. Furthermore, sequencing and characterizing of valuable markers may be required for better trait selection for breeders' attention, perhaps improving this crop through marker assisted selection. The current study's findings will be useful for DNA fingerprinting and analyzing genetic variances among a wide array of cultivars. Understanding the degree of genetic link between different cultivars is critical for germplasm collection, *in situ* conservation, and grapevine breeding operations.

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Ethical approval
This study does not involve human/animal subjects, and thus no ethical approval is needed.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of the experiment, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All authors have critically read this manuscript and agreed to publish in IJAaEB.

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