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Molecular Characterization of Grape Genetic Resources Utilizing Simple Sequence Repeat (SSR) Markers

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Abstract **– The objective of this study was molecular characterization of 27 grape accessions maintained at the ICAR–National Bureau of Plant Genetic Resources, Regional Station, Shimla, Himachal Pradesh, India using 9 SSR primer pairs. The accessions contained 16 Vitis vinifera cultivars, 6 non-vinifera species and their cultivars, 4 inter-specific hybrids, and 1 non-Vitis genus (Parthenocissus quinquefolia) from within the Vitaceae family. SSR fragments were analyzed on 3500 Genetic Analyzer (ABI). A total of 89 distinct alleles were scored in 27 grape accessions, and the average number of alleles per locus was 9.89. The polymorphism information content (PIC) ranged from 0.7734 to 0.8922 with a mean of 0.8532. The clustering dendrogram could differentiate all grape accessions and divide them into 5 divergent multi-genotypic clusters. Clustering of the accessions was not specific to species categories, indicating the complex origin of some of the accessions. The result of the factorial analysis was also in agreement with the result of hierarchical clustering. In addition to the development of a molecular database, our results indicated that the studied grape genetic resources represent a genetically rich and diversified genetic resource that can be used as a valuable material for future scion and root stock improvement programmes in India.**

Keywords **– Vitis Vinifera, Grapevine, Microsatellite, Germplasm, Genetic Diversity.**

I. INTRODUCTION

The grape (*Vitis vinifera* L.), one of the most important fruit crops in the world, is grown in a wide range of environments (Liu et al., 2012). Taxonomists consider the regions between the south of the Caspian and Black seas in Asia Minor to be the original home of the old-world grape, *Vitis vinifera* L. (Snyder, 1937). From here, the grape has spread in all temperate climates and more recently in tropical and subtropical regions. It is believed that the grape was introduced to India in 1300 AD by the invaders from Iran and Afghanistan (Thaper, 1960). Thereafter, grape cultivation spread in different parts of the country, and currently, it is more concentrated in Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu. In 2020, among the 10 major grape producing countries of the world, India ranked 9th in area, 7th in production and 1st in grape productivity. In the same year, India produced 3.125 million tonnes of grapes from an area of 0.14 million ha with an average productivity of 22.32 t/ha (FAOSTAT, 2022). The highest grape productivity in India is mainly due to adoption of an effective canopy management and modern production technologies. Currently, Thompson Seedless (with its clones) is the ruling grape variety in India, occupying about 55% of the area followed by Bangalore Blue which occupies approximately 15% area, while, the remaining 30% area is covered by Anab-e-Shahi & Dilkhush (15%), Sharad Seedless (5%), Perlette (5%), and Gulabi & Bhokri (5%) (APEDA, 2022). Most of these varieties are either exotic introductions or their clonal selections. However, for retaining and further enhancing high grape productivity in India, efforts are needed to develop multipurpose, climate resilient, and high yielding improved grape cultivars (scions) as well as robust root stocks.

Characterization and diversity studies on available grape genetic resources and land races are essential for their proper documentation, conservation, and utilization in breeding programmes. Traditionally, morphological characters have been used for the characterization of grape genetic resources. This analysis is a fast and inexpensive method; however, the morphological characters are strongly influenced by the environment, devoid of the resolving power due to scarcity of scorable attributes and their interpretation is sometimes subjective, thus, reliable discriminations among morphologically similar genetic resources are difficult (Martinez et al., 2008). Characterization and diversity estimation by biochemical markers, viz. isozyme analysis, may also be biased as only a minor portion of the genome is represented by these markers and they are also influenced by the environment and metabolic state of the organism/tissue (Noori et el., 2010). Molecular DNA markers, on the other hand, are not influenced by the environment and their interpretation is more objective. Therefore, they are a valuable alternative for discriminating closely related genotypes. Among them, random amplified polymorphic DNA (RAPD) (Lima et al., 2006), restriction fragment length polymorphism (RFLP) (Bowers and Meredith, 1996), amplified fragment length polymorphism (AFLP) (Upadhyaya et al., 2007), and simple sequence repeat (SSR) (Bowers et al. 1996; Sefc et al. 2000) have been used in genetic diversity studies in grapevine. Among them, simple sequence repeats (SSRs) or microsatellites are markers of choice for the molecular characterization of grapes because of their procedural ease, robustness, polymorphism, reproducibility, and codominant nature (Sefc et al., 2001; This et al. 2004). Many SSR markers are being used by the researchers in grapes to access genetic diversity. However, six markers viz., VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79 are called a “core set” markers that are highly recommended for comparison of results in different laboratories (Sefc et al., 2001; This et al., 2004). In addition to these 6 core markers, International Organisation of Vine and Wine (OIV) added 3 more SSR markers viz., VVMD25, VVMD28 and VVMD32 to the OIV register for identification and characterization of grape genotypes (OIV, 2009).

A valuable collection of grape varieties, root stocks and wild species is maintained at the ICAR-National Bureau of Plant Genetic Resources, Regional Station, Shimla, Himachal Pradesh, India. The objective of the present study was to utilize core set of above 9 SSR markers for molecular characterization of these grape genetic resources.

II. Materials and Methods

Young fresh leaves having uniform size and freedom from insects and diseases were collected from 27 grape accessions maintained at the ICAR-National Bureau of Plant Genetic Resources, Regional Station, Shimla, Himachal Pradesh, India (31.097786 N; 77.160409 E; 1876 msl). The leaf samples were packed in aluminium foil and stored in zipper bags at -20 ^{0}C till further use. The accessions contained 16 *Vitis vinifera* cultivars, 6 non-*vinifera* species and their cultivars, 4 inter-specific hybrids, and 1 non-*Vitis* genus (*Parthenocissus quinquefolia)* from within the Vitaceae family (Table 1).

Table 1. List of grape accessions used for molecular characterization.

Genotype Code	Accession Number	Name of the Accession
PGR-01	EC772083	*Vitis riparia x (V. cordifolia x V. rupestris)*/ Malegue 44-53
PGR-02	EC772100	*Vitis vinifera*/ Mauzac
PGR-03	EC772085	*Vitis vinifera*/ Admirable de Curtiller

Genotype Code	Accession Number	Name of the Accession
PGR-04	EC772082	*Vitis riparia x V. rupestris*/ Couderc 3309
PGR-05	EC732195	*Vitis interspecific cross*/ Black Fredonia Bunch
PGR-06	EC772103	*Vitis vinifera*/ Muscat A Petits Grains Blancs
PGR-07	EC772092	*Vitis vinifera*/ Chardonnay
PGR-08	EC452213	*Vitis amurensis*/ DVIT2005.5
PGR-09	EC772096	*Vitis vinifera*/ Hans
PGR-10	EC452207	*Vitis arizonica*/ DVIT1269
PGR-11	EC452206	*Vitis ficifolia*/ DVIT1106
PGR-12	EC452209	*Vitis berlandieri*
PGR-13	EC772086	*Vitis vinifera*/ Aghiorghitiko
PGR-14	EC772094	*Vitis vinifera*/ Furmint
PGR-15	EC772087	*Vitis vinifera*/ Alvarelhao
PGR-16	EC772106	*Vitis vinifera*/ Touriga Nacional
PGR-17	EC772095	*Vitis rupestris*/ Goethe 9
PGR-18	EC772098	*Vitis vinifera*/ Madeleine Royale
PGR-19	EC452215	*Parthenocissus quinquefolia*/ DVIT2400
PGR-20	EC772108	*Vitis vinifera*/ Savagnin Rose
PGR-21	EC772090	*Vitis vinifera*/ Cabernet Sauvignon
PGR-22	EC772101	*Vitis vinifera*/ Merlot
PGR-23	EC732197	*Vitis labrusca*/ Niagara Bunch
PGR-24	EC772080	*Vitis longii x V. riparia*/ Couderc 1616
PGR-25	IC566150	*Vitis vinifera*/ Unknown variety
PGR-26	EC772099	*Vitis vinifera*/ Mancin
PGR-27	EC772107	*Vitis vinifera*/ Ugni Blanc

DNA was extracted from the leaf tissues based on a modified CTAB-dichloromethane protocol described by Saghai-Maroof et al. (1984). Details of the SSR markers used in the present study are given in Table 2. PCR was performed in 25 µl reaction volume containing 2.5 µl of 10x PCR buffer (without $MgCl_2$), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 µM of each forward and reverse primer, 0.5 µl of 10mM dNTP, 0.2 µl of 5 U/µl of Taq polymerase (Qiagen, Venlo, Limburg, Netherlands), about 50 ng of template genomic DNA and Milli-Q water to make reaction volume to 25 µl. The PCR mixtures were cycled at 94°C for 1 min, 50-55 °C (depending on primer) for 1 min, and 72°C for 1 min repeated for 35 cycles on a Veriti Thermal Cycler (Life Technologies, Carlsbad, CA, USA). Genetic analyzer [3500 Genetic Analyzer (ABI)] was used to resolve FAM labelled SSR amplified PCR products. Reactions were set up in 96 well plate (ABI), each well containing 12 µl Hidi

formamide (ABI), 3 µl amplified PCR product and 0.4 µl GeneScan-500 ROX (ABI). The PCR products were denatured at 95 ^{0}C for 5 minutes followed by quick chilling on ice. The ABI plate was loaded in "3500 Genetic Analyzer" and run as per manufacturer's instructions to separate PCR amplified products by capillary electrophoresis. Capillary electrophoresis data were converted into allelic sizes using GeneMapper Software Version 4.1 (Applied Biosystems, California, USA). Fragment calibration was performed with an internal size standard GeneScan-500 ROX.

Table 2. Information on SSR markers used in this study.

SN	Marker*	Forward (F) and Reverse (R) Primer Sequences (5' -3')	TA** (^{0}C)	No. of Alleles	Allele Size Range (bp)
1	VVS-2	F: CAGCCCGTAAATGTATCCATC	50.0	13	123-162
		R: AAATTCAAAATTCTAATTCAACTGG			
2	VVMD5	F: CTAGAGCTACGCCAATCCAA	56.0	8	226-246
		R: TATACCAAAAATCATATTCCTAAA			
3	VVMD7	F: AGAGTTGCGGAGAACAGGAT	52.0	11	233-263
		R: CGAACCTTCACACGCTTGAT			
4	VVMD25	F: TTCCGTTAAAGCAAAAGAAAAAGG	56.0	11	243-275
		R: TTGGATTTGAAATTTATTGAGGGG			
5	VVMD27	F: GTACCAGATCTGAATACATCCGTAAGT	56.0	11	173-194
		R: ACGGGTATAGAGCAAACGGTGT			
6	VVMD28	F: AACAATTCAATGAAAAGAGAGAGAGAGA	56.0	15	221-279
		R: TCATCAATTTCGTATCTCTATTTGCTG			
7	VVMD32	F: TATGATTTTTTAGGGGGGTGAGG	56.0	11	239-273
		R: GGAAAGATGGGATGACTCGC			
8	VrZAG62	F: GGTGAAATGGGCACCGAACACACGC	58.0	11	185-203
		R: CCATGTCTCTCCTCAGCTTCTCAGC			
9	VrZAG79	F: AGATTGTGGAGGAGGGAACAAACCG	58.0	13	236-260
		R: TTGGAAGTAGCCAGCCCAACCTTC			

*References: SN 1: Thomas and Scott (1993); SN 2 & 3: Bowers et al. (1996); SN 4-7: Bowers et al. (1999); SN 8 & 9: Sefc et al. (1999) ** TA = Annealing Temperature.

DARwin 6.0.21 (Perrier et al., 2003) software was used for data analysis. The PIC value of SSR marker was calculated according to the formula: PIC = 1−Σ (P2i), where Pi is the frequency of the ith allele of a marker detected in accessions (Nei, 1973). Jaccard coefficient of dissimilarity, 100 bootstraps and weighted neighbor joining were used to construct radial tree using DARwin 6.0.21 software. Factorial analysis was also conducted using DARwin 6.0.21.

III. Results and Discussion

Molecular markers exhibiting polymorphism at the DNA level are highly useful tools for the characterization

of germplasms (Queiroz et al., 2015). Allelic composition of genetically diverse grape accessions was revealed using a core set of 9 SSR primers is given in Table-3. The nine SSR markers used in our study, are highly polymorphic, have been used by the European GrapeGen06 consortium (Maul et al., 2012) and frequently used to identify grape cultivars (This et al. 2004; and Li et al. 2018). Internationally, France, Germany, Italy, and other countries are also using these SSR markers to establish molecular databases of grape cultivars (Maul and Topfer, 2015). DNA from all 27 accessions was successfully amplified using these SSRs. A total of 89 alleles, with sizes between 119 and 271 base pairs, were obtained (Table-3). Obtained allele sizes are in agreement with those of Stajner et al. (2011) (allele sizes 131 bp to 274 bp); Rao et al. (2014) and Alba et al. (2015) (allele sizes 123 bp to 268 bp); and Bibi et al. (2020) (allele sizes 121 bp to 289 bp). In our studies, the number of alleles per locus ranged from 8 to 13 with an average of 9.89 alleles per locus. The observed average number of alleles per locus are in agreement with 9.8 and 9.5 alleles/per locus reported by Stajner et al. (2011) and Alba et al. (2015), respectively. While, characterizing 100 grape accessions containing 23 wild species, Li et al. (2021) reported an average of 20.67 alleles/locus. It appears that average number alleles per locus is more when genetically diverse genotypes are subjected to molecular analysis. Polymorphic information content (PIC) values varied from 0.7734 in VVMD5 to 0.8922 in VVMD28 with a mean of 0.8532 (Table-4). The observed mean PIC value was higher than mean PIC values reported by Stajner et al. (2011) (0.776) and Bibi et al. (2020) (0.7379). However, much higher average PIC value (0.903) has been reported by Li et al. (2021) for 100 grape accessions containing 23 wild species. High PIC value suggests a high discriminating ability of the SSR markers and it appears that higher PIC values are exhibited by genetically diverse genotypes.

Table 3. Database of SSR allelic variations in 27 grape genetic resources.

Genotype	SSR Marker									Allele Frequency
	VVS2	VVMD5	VVMD7	VVMD25	VVMD27	VVMD28	VVMD32	VrZAG62	VrZAG79	
PGR-01	119, 139, 145	247, 262	233, 239	240, 242	208, 212	227, 257	200, 235, 251	180, 185	256, 264	20
PGR-02	134, 153	233, 235	237, 249	250	186, 191	227, 235	239, 241	187, 200	250	16
PGR-03	124	224, 233, 237	237, 239	242, 256	180, 191	235, 268	235, 239	187, 193	248, 254	18
PGR-04	119, 124	247, 262	247, 257	237, 240	186, 212	240, 247	235, 239	180, 189	254, 256	18
PGR-05	119, 124, 127	237	235	242	184, 186	227, 245	271	203	246, 258	14
PGR-06	134	227, 237	233, 249, 253	240, 250	182, 196	245, 268	249, 262, 271	187, 195	250, 254	19
PGR-07	139, 142, 153	233, 237	237, 243	240, 256	182, 191	218, 227, 231	239, 271	187, 195	242, 246	20
PGR-08	119	224, 227	235, 239, 249	242, 250	178, 180, 182	Nil	249, 251	187, 193	258, 264	17
PGR-09	127, 130, 134	235, 247	249, 253	237, 240	186, 191	237, 268	249, 262	193, 203	250	18
PGR-10	119	233, 235	249, 253	240	180, 182	Nil	Nil	Nil	Nil	8
PGR-11	119	235	239, 241	240, 242	196, 202	227, 231	200, 235	187	236, 246	15

Genotype	SSR Marker									Allele Frequency
	VVS2	VVMD5	VVMD7	VVMD25	VVMD27	VVMD28	VVMD32	VrZAG62	VrZAG79	
PGR-12	134, 145	247	239	242, 250	196	237	235	200	233, 236	12
PGR-13	119	235	233, 266	237, 240	202, 208, 212	221, 245	235, 247	187, 195	250, 258	17
PGR-14	134, 153	224, 239	239, 247, 249	240, 242	180, 196	227, 249	262, 271	187, 203	236, 250	19
PGR-15	134, 153	224, 227	237, 239	240, 250	186, 191	235, 259	241, 256	187, 193	240, 246	18
PGR-16	145, 153	227, 239	239	240, 250, 256	182, 191	235, 268	239, 271	189, 193	242, 246	18
PGR-17	139, 142	262	249, 266	237, 246	186, 212	237, 240	239	200	258	14
PGR-18	119, 149, 153	227, 237	243, 247	240, 250	182, 191	218, 245	251, 271	187, 195	246, 258	19
PGR-19	139	Nil	Nil	Nil	176	Nil	Nil	Nil	236	3
PGR-20	149, 153	235, 239	243, 257	250	191	235, 237	239, 271	189, 195	246, 250	16
PGR-21	139, 142, 153	233, 239	239	240, 250	176, 191	235, 237	239	187, 193	246	16
PGR-22	149, 153	227, 237	239, 243, 247	250	191, 196	227, 247	239, 271	193, 195	250, 258	18
PGR-23	124, 134	237	235, 241	240, 242	180, 186	231, 237	247, 271	200, 203	236, 258	17
PGR-24	139, 142	262	237, 239, 249	240, 242	196, 212	245, 249	235, 239	189, 193	254, 258	18
PGR-25	149, 153	235	237, 243	242, 246	176, 182	268	256, 271	193, 195	242, 250	16
PGR-26	119, 139, 153	235, 237	239	240, 256	174, 191	249, 268	239, 241, 256	187, 193	246, 250	19
PGR-27	119, 134, 142	227, 233	243, 253	237, 250	182, 191	268	271	200, 203	240, 258	17
Allele Frequency	55	45	52	48	53	46	48	46	47	440

Table 4. Allelic variation and polymorphic information content of SSR markers used in analysis of grape accessions.

SN	Primer Name	SSR Alleles		PIC [b]
		Number	Size (bp) and Absolute Allele Frequencies [a]	
1	VVS2	10	119(10), 124(4), 127(2), 130(1), 134(8), 139(7), 142(5), 145(3), 149(4), and 153(11)	0.8661
2	VVMD5	8	224(4), 227(7), 233(6), 235(8), 237(8), 239(4), 247(4), and 262(4)	0.8632
3	VVMD7	11	233(3), 235(3), 237(6), 239(12), 241(2), 243(6), 247(4), 249(8), 253(4), 257(2), and 266(2)	0.8735
4	VVMD25	6	237(5), 240(16), 242(10), 246(2), 250(11), and 256(4)	0.7734
5	VVMD27	12	174(1), 176(3), 178(1), 180(5), 182(8), 184(1), 186(7), 191(12), 196(6), 202(2), 208(2),	0.8708

SN	Primer Name	SSR Alleles		PIC [b]
		Number	Size (bp) and Absolute Allele Frequencies [a]	
			and 212(5)	
6	VVMD28	13	218(2), 221(1), 227(7), 231(3), 235(6), 237(6), 240(2), 245(5), 247(2), 249(3), 257(1), 259(1), and 268(7)	0.8922
7	VVMD32	10	200(2), 235(7), 239(11), 241(3), 247(2), 249(3), 251(3), 256(3), 262(3), and 271(11)	0.8507
8	VrZAG62	8	180(2), 185(1), 187(12), 189(4), 193(10), 195(7), 200(5), and 203(5)	0.8280
9	VrZAG79	11	233(1), 236(5), 240(2), 242(3), 246(9), 248(1), 250(9), 254(4), 256 (2), 258(9), and 264(2)	0.8610
	Total	89		7.6789
	Average	9.89		0.8532

[a] Absolute allele frequencies in parentheses represent the actual number of samples (out of total 27) in which amplification was observed. [b] PIC: Polymorphic Information Content.

The factorial analysis conducted using DARwin software indicated that 30.45% of the total variance was explained by the first three components 13.38%, 9.33%, and 7.74%, respectively. A factorial graph depicting the distribution and diversity of grape accessions is shown in Figure-1. Significant genetic diversity among studied grape accessions was also exhibited by the dendrogram generated using DARwin software (Figure-2). All 27 accessions could be successfully discriminated. The dendrogram exhibited 5 clusters. Cluster I contained 3 *V. vinifera* cultivars, *V. ficifolia*, *V. berlandieri* and *Parthenocissus quinquefolia*; cluster II had 2 *V. vinifera* cultivars and 3 interspecific hybrids; cluster III contained 3 *V. vinifera* cultivars, *V. arizonica*, and *V. amurensis*; and cluster IV and V contained 11 *V. vinifera* cultivars. Though the findings indicated relative relationships among studied grape accessions, grouping of genetically diverse accessions in some of the clusters suggested complex origins of some of these accessions.

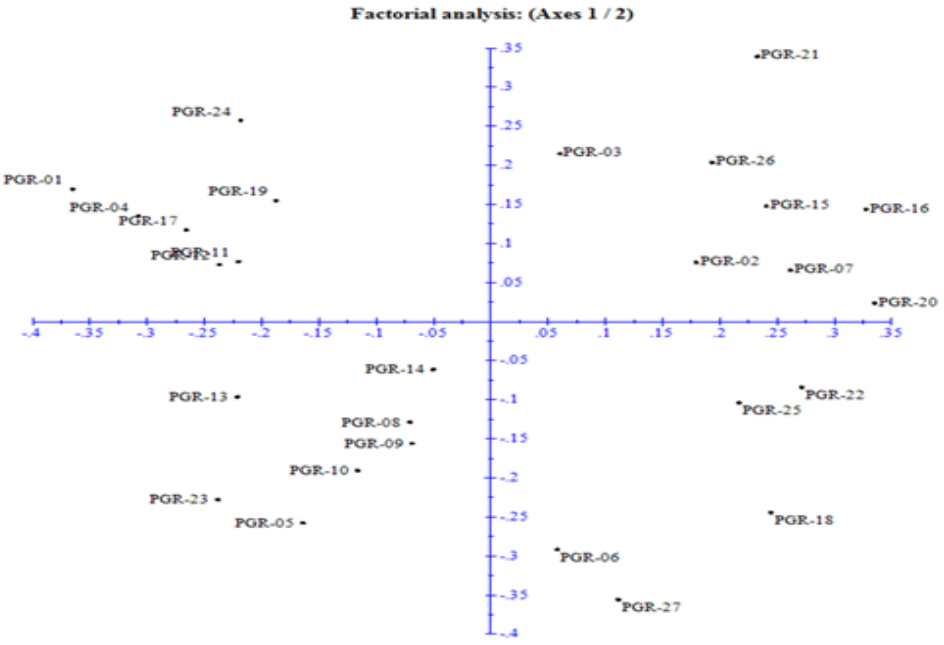

Fig. 1. Factorial analysis of 27 grape accessions using SSR profiles and DARwin 6.0.21 software (Jaccard dissimilarity index and 100 bootstraps).

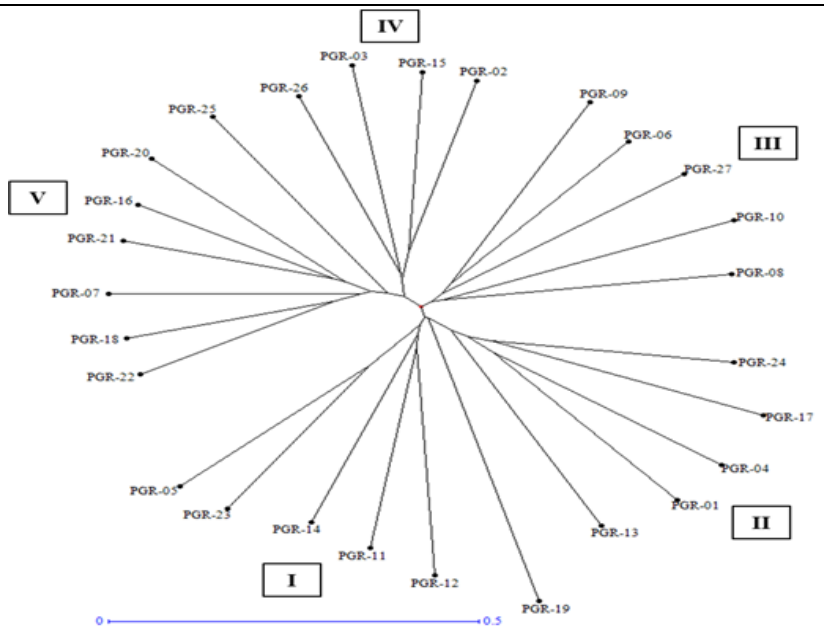

Fig. 2. Clustering of 27 grape accessions using SSR profiles and DARwin 6.0.21 software (Jaccard dissimilarity index and 100 bootstraps).

IV. Conclusion

In addition to the molecular characterization of grape accessions and development of molecular database, our results conclude that grape genetic resources maintained at the ICAR-National Bureau of Plant Genetic Resources, Regional Station, Shimla, Himachal Pradesh, India represent a genetically rich and diversified genetic resource that can enter as valuable material for future sustainable breeding and improvement of grapevine.

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