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GENETIC DIVERSITY ANALYSIS OF GINGER GERMPLASM OF SOUTH RAJASTHAN THROUGH SCoT AND ISSR PRIMERS

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Ginger (*Zingiber officinale*) is a well-known spice herb showing anti-inflammatory, antitumour, antioxidant, antiapoptotic, cytotoxic and antiproliferative activities due to the occurrence of a diverse range of phytochemicals. Ginger is dominantly cultivated in many districts of South Rajasthan due to favourable climatic and soil conditions but the yield is very low as the crop is highly susceptible to various bacterial and fungal diseases. The unavailability of resistant ginger varieties resulted in a sharp decline in ginger cultivation. Genetic diversity analysis is desirable to develop improved varieties of ginger showing resistance against biotic and abiotic stress. The present paper reports the assessment of genetic diversity among 30 ginger genotypes from South Rajasthan through SCoT and ISSR markers. An overall 62.93% polymorphism was reported by the combined use of SCoT and ISSR markers and SCoT markers performed better in terms of the number of amplification products (168 bands) and a higher degree of polymorphism (74.39%). Cluster analysis confirmed that genotypes which were collected from a similar geographical area are genetically similar and diversity arose due to a slight change in geographical location. Population genetic analysis revealed that members of the DOD population showed the highest genetic diversity while AMI and SAV populations are closely related. The present paper proved the potential of SCoT markers for the evaluation of genetic diversity in other ginger accessions as well as for the identification of positive traits for varietal improvement.

Keywords: Genetic diversity, ISSR, Molecular markers, SCoT, Spice.

Introduction

Ginger (*Zingiber officinale*) is known to the entire world as an important herbal supplement that has tremendous culinary and medicinal uses. Ginger contributes to 5-6% of total spices consumed worldwide. It is a monocot plant belonging to the family Zingiberaceae. Mainly the rhizome, both fresh as well as dry, has been used since ancient times to treat conditions like cough and cold, fever, vomiting and nausea, digestive disorders, arthritis, migraines, hypertension etc. as it contains several biologically active compounds such as shogaol, gingerol, ginger protease, capsaicin and several sesquiterpenes like zingiberenol, zingiberol[1]. Chemical analysis proved that ginger contains more than 400 compounds including carbohydrates, lipids and terpenes as major constituents while amino acids, proteins, vitamins, minerals, raw fibres and phytosterols are present in low[2,3]. Primary terpenes of ginger are monoterpenoids (β-phellandrene, (+)-camphene, cineole, geraniol etc.) and sesquiterpenoids (α-zingiberene, β -sesquiphellandrene, β-bisabolene, (E-E)- α -farnesene, arcurcumene, and zingiberol)[4].

These active principles impart characteristic pungency, order and flavourto ginger and are responsible for a range of biological and pharmacological effects like anti-inflammatory, antitumour, antioxidants, antiapoptotic, cytotoxic and antiproliferative. Therefore, a huge market developed globally for ginger. As per the EMR report, 2023, the export value of ginger is approximately 884.5 million US $ out of which 31.4 million US $ value ginger is exported from India[5]. Green ginger is mainly exported to European countries while other ginger products like dry ginger, ginger powder, ginger paste, and ginger oleoresins are traded globally including USA, UAE, UK, Saudi Arabia and Nepal as the top five importer countries. Ginger is cultivated in the tropical and sub-tropical countries of the world including India and China as leading producer countries.

India is the largest ginger grower country and producer of dry ginger around the world and contributes 30% of the world's total production. Although ginger is grown almost in every state of India, Madhya Pradesh, Karnataka, Assam, Maharashtra and Rajasthan are dominating states where ginger farming is practised by the tribal community. Three districts of South Rajasthan namely Udaipur, Dungarpur and Baran contribute a major proportion of the total ginger cultivation (246 tons in 122 ha). Farmers of South Rajasthan are interested in ginger cultivation as they are aware that the climatic conditions of South Rajasthan are favourable to growing ginger and the crop is extensively used as spice and medicine throughout the world. For them, ginger cultivation is more profitable compared to the cultivation of other regional crops but the production is low and it is decreasing day by day[6]. The ginger crop is very susceptible to bacterial and fungal pathogens and suffers poor pre-harvest and post-harvest disease management practices. Therefore, the lack of disease-resistant, higher-yielding varieties of ginger might be a reason for low production[7,8]. The overall crop yield hence, the profit of farmers is significantly reduced due to these unsolved problems which withdrew the farmer's interest in ginger farming. Therefore, the analysis of genetic diversity in ginger is essential as it leads to the identification of superior alleles which code for important traits such as higher yield, improved percentage of essential oils, better flavour and taste etc, and availability of genetically distinct clones which will be used for varietal improvement programs to develop disease-resistant varieties of ginger.

Molecular markers proved to be very promising for genetic diversity analysis in plants as they identify and characterise plants through direct access to the genome. Additionally, they are not affected by changing environmental factors[9]. The information obtained through molecular marker-based genetic diversity assessment consequently be used in designing a strategy for the development of novel varieties of ginger showing resistance against biotic or abiotic stress. Molecular markers have been widely used in other crops to assess genetic diversity, but very few reports are there on genetic diversity analysis in ginger by using various types of non-coding molecular markers such as AFLP, RAPD, ISSR, and microsatellite (SSR). Although South Rajasthan is a well-known ginger-growing region, little attention has been paid to the molecular characterisation of the available ginger germplasm, although it is a significant source of income for the local people. Therefore, a more informative, robust marker is needed for ginger as previous studies were unable to correlate the low genetic variability with more diversity in morphological features. The present paper describes the use of SCoT (start codon targeted) and ISSR markers to assess the genetic diversity in ginger genotypes. SCoT markers are designed by targeting the start codon of protein-coding genes in the genome. Thus, they differ meaningfully

from previously implemented non-coding DNA-based markers.

Material and Methods

Collection of plant material

Multiple ginger fields in different villages of Udaipur and Pratapgarh districts were visited in the rainy season to collect juvenile, disease-free ginger leaves. The sample collection sites and collected samples were coded appropriately and shown in Table-1. Random samplings were performed in each site and the collected leaves were used for the extraction of genomic DNA.

Table1.: Information about ginger samples that were used in the present study.

S. No.	Population location, Latitude longitude, elevation	Site acronym	Samples code name
1	Amiwara, Jhadol, Udaipur. 24.2956° N, 73.3753° E, 359 Meters	AMI	G1, G2, G3
2	Pratappura, Choti sadri, Pratapgarh. 27.1660° N, 78.0092° E, 154 Meters	PRA	G4
3	Bansiwara, Jhaol, Udaipur. 24.3984° N, 73.3031° E, 302 Meters	BAN	G5, G6, G7
4	Rohimala, Jhadol, Udaipur. 24.3973° N, 73.3443° E, 359 Meters	ROH	G8
5	Savina, Udaipur. 24.5476° N, 73.7095° E, 540 Meters	SAV	G9, G10, G11
6	Looniyara, Jhadol, Udaipur. 24.36149° N, 73.40043° E, 359 Meters	LOO	G13, G14, G15, G16, G17, G18
7	Jetawara, Jhadol, Udaipur. 24.5881° N, 72.3163° E, 227 Meters	JET	G19, G20, G21, G22, G23, G24
8	Dodawali, Girwa, Udaipur. 24.5620° N, 73.5535° E, 540 Meters	DOD	G12, G25, G26, G27, G28, G29, G30

Extraction of genomic DNA

The young leaves of ginger were used for the extraction of high-quality, intact genomic DNA (gDNA). Before beginning with the DNA extraction phase, collected ginger leaves were frozen in liquid nitrogen (-196℃) and pulverised into a fine powder by grinding them in a sterilised mortar and pestle. The CTAB method with slight modifications was used for DNA extraction from the prepared leaf powder. Approximately 1 g of leaf powder was placed in a centrifuge tube (14 ml, Tarson) and 5 ml of freshly prepared pre-heated (65 °C) DNA extraction buffer (100 mM Tris HCl, 4 M NaCl, 20 mM EDTA, 50 mM ascorbic acid, 2% CTAB, 2% PVP and 0.2% β-mercaptoethanol) was added to it. The tubes containing samples were swirled gently for 8–10 min then incubated in a water bath at 65 °C for 45 min with intermittent swirling. After incubation, the homogenates were brought to room temperature and subjected to further steps as per the protocol[10,11]. Finally, the obtained DNA pellet was dried and re-suspended in 1000 µl of sterile H_2O. The quantity of isolated gDNA was measured with a Quantus fluorometer by using a fluorescent dye. High-quality genomic DNA was observed with no RNA contamination, having an average yield of 0.25 µg/µl.

DNA amplification

All DNA amplification reactions were performed in 25 µl volume containing 50 ng of template DNA, 1X PCR buffer, 0.6 µM of primer, 2500 µM of each dNTP, 25 mM $MgCl_2$ and 1.5 units of Taq DNA polymerase (Bangalore GeNei brand).

Table 2.: Details of SCoT and ISSR primers used in present study.

S. N.	Primer name	Primer sequence 5'_3' (length)[a]	Melting temperature (Tm) ℃	Annealing temperature (Ta) ℃	Total no. of bands	No. of polymorphic bands	% polymorphism
		SCoT primers					
1	SCoT-2	CAACAATGGCTACCACCC (18 mers)	53.60	51.00	16	13	81.25
2	SCoT-3	CAACAATGGCTACCACCG (18 mers)	53.90	51.00	18	12	66.67
3	SCoT-4	CAACAATGGCTACCACCT (18 mers)	52.30	50.00	14	7	50.00
4	SCoT-5	CAACAATGGCTACCACGA (18 mers)	52.60	50.00	11	6	54.55
5	SCoT-6	CAACAATGGCTACCACGC (18 mers)	54.40	52.00	16	13	81.25
6	SCoT-7	CAACAATGGCTACCACGG (18 mers)	53.90	51.00	12	7	58.33
7	SCoT-8	CAACAATGGCTACCACGT (18 mers)	52.90	50.00	15	13	86.67
8	SCoT-9	CAACAATGGCTACCAGCA (18 mers)	52.90	50.00	14	13	92.86
9	SCoT-10	CAACAATGGCTACCAGCC (18 mers)	53.90	51.00	7	5	71.43
10	SCoT-12	ACGACATGGCGACCAACG (18 mers)	58.40	56.00	11	9	81.82
12	SCoT-13	ACGACATGGCGACCATCG (18 mers)	58.00	56.00	20	15	75.00
13	SCoT-15	ACGACATGGCGACCGCGA (18 mers)	62.60	60.00	14	13	92.86
		ISSR primers					
1	UBC-808	AGA GAG AGA GAG AGA GC (17 mers)	48.80	46.00	13	5	38.46
2	UBC-811	GAG AGA GAG AGA GAG AC (17 mers)	46.80	44.00	15	5	33.33
3	UBC-812	GAG AGA GAG AGA GAG AA (17 mers)	45.70	43.00	11	2	18.18
4	UBC-823	TCT CTC TCT CTC TCT CC (17 mers)	48.10	46.00	11	5	45.45
5	UBC-818	CAC ACA CAC ACA CAC AG (17 mers)	51.00	49.00	9	8	88.89
6	UBC-825	ACA CAC ACA CAC ACA CT (17 mers)	51.40	49.00	7	3	42.86
7	UBC-826	ACA CAC ACA CAC ACA CC (17 mers)	52.80	50.00	15	8	53.33
8	UBC-841	AG AGA GAG AGA GAG A(CT)C (18 mers)	49.90	47.00	11	5	45.45

Amplification was carried out in a thermocycler (Eppendorf 5331) with initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at Ta °C for 1 min and elongation at 72°C for 1 min. A final extension cycle at 72°C for 10 min was followed. Later the amplification products were kept on hold at 4°C to stop the amplification reaction. The annealing temperature (Ta) was kept at 2°C below the melting temperature (Tm) of a particular primer. All SCoT and ISSR primers with desired sequences were

synthesised from IDT (Integrated DNA Technologies Inc., USA). Therefore, information about their melting temperature (Tm) was retrieved from the IDT website[12]. Table-2 summarises the information about the selected primers. All amplification products were resolved on 1.5% agarose gel containing ethidium bromide (0.25 µg/ml). The gel was visualised and photographed through a Bio-Rad Gel Doc XR^+ imaging system (Bio-Rad Laboratories India Pvt. Ltd.). Initially, single genotype of ginger (G1) was amplified by using 15 SCoT and 10 ISSR markers to analyse their amplification ability. Based on the results of preliminary screening 12 SCoT and 8 ISSR primers which gave clear amplification with reproducible bands were used for polymorphism studies.

Table-3.: Genetic diversity statistics and differentiation parameters for eight populations of *Z. officinale*.

Population	N	Na ± SD	Ne ± SD	h ± SD	I ± SD	Pp (%)	G_{ST}	Nm
AMI	3	1.0885 ± 0.2845	1.0601 ± 0.2085	0.0345 ± 0.1144	0.0508 ± 0.1662	8.85		
PRA	1	1.0000 ± 0.0000	1.0000 ± 0.0000	1.0000 ± 0.0000	1.0000 ± 0.0000	0.00		
BAN	3	1.0962 ± 0.2954	1.0613 ± 0.2047	0.0361 ± 0.1144	0.0537 ± 0.1679	9.62		
ROH	1	1.0000 ± 0.0000	1.0000 ± 0.0000	1.0000 ± 0.0000	1.0000 ± 0.0000	0.00		
SAV	3	1.1423 ± 0.3500	1.0993 ± 0.2636	0.0564 ± 0.1432	0.0828 ± 0.2072	14.23		
DOD	7	1.6115 ± 0.4883	1.3758 ± 0.3836	0.2179 ± 0.2016	0.3254 ± 0.2860	61.15		
LOO	6	1.2385 ± 0.4270	1.1213 ± 0.2663	0.0736 ± 0.1480	0.1134 ± 0.2181	23.85		
JET	6	1.1731 ± 0.3790	1.0696 ± 0.1917	0.0453 ± 0.1124	0.0728 ± 0.1713	17.31		
	30	1.7231 ± 0.4483	1.2775 ± 0.3132	0.1769 ± 0.1680	0.2829 ± 0.2389	72.31	0.6393	0.2821

*N, sample size; Na, observed number of alleles; Ne, effective number of alleles [25]; h, [26]gene diversity; I, Shannon's information index [27]; Pp, percentage of polymorphic loci [28]; SD, standard deviation; GST, diversity among populations; Nm, geneflow 0.5(1−GST)/GST; FST, fixation index. Parameter calculations assume Hardy–Weinberg equilibrium.

Cluster analysis

Each amplification product was evaluated across all samples as a potential DNA marker. Scoring of gel was done manually by designating '1' for the presence and '0' for the absence of a particular amplification band. All the faint and low-intensity bands were not considered during the final scoring. Data for SCoT and ISSR primers were combined to prepare a binary matrix. The Jaccard coefficient[13] was used by the FreeTree programme version 0.9.1.5 to create a pair-wise matrix of distances between genotypes[14]. After enabling a bootstrap test of 1000 iterations using the same algorithm, a single Neighbour-Joining (NJ) tree was computed using this distance matrix. A consensus tree was chosen based on a majority rule. FigTree version 1.2.2 was used to view, annotate, and print the tree. Relationships among populations were deciphered through Nei's [15] unbiased population genetic distance by POPGENE version 1.31. by using cumulative data of SCoT and ISSR markers[16].

Population genetic analysis

With POPGENE version 1.31[16], genetic diversity parameters such as the proportion of polymorphic loci (Pp), Nei's gene diversity (h), and Shannon's information index (I) were calculated to determine the level of genetic variation. The geneflow (Nm) was calculated using the equation $Nm = 0.5(1-G_{ST})/G_{ST}$.

Results and Discussion

Profile polymorphism

Thirty ginger samples from two districts of South Rajasthan (Udaipur and Pratapgarh which are the well-known ginger growing districts) were assessed for genetic diversity using SCoT and ISSR markers. A total of

260 amplification products were scored among which 168 were obtained from SCoT and 92 were obtained from ISSR. An overall polymorphism of 62.93% was found, with SCoT showing 74.39% and ISSR showing 45.75% (Table 2).

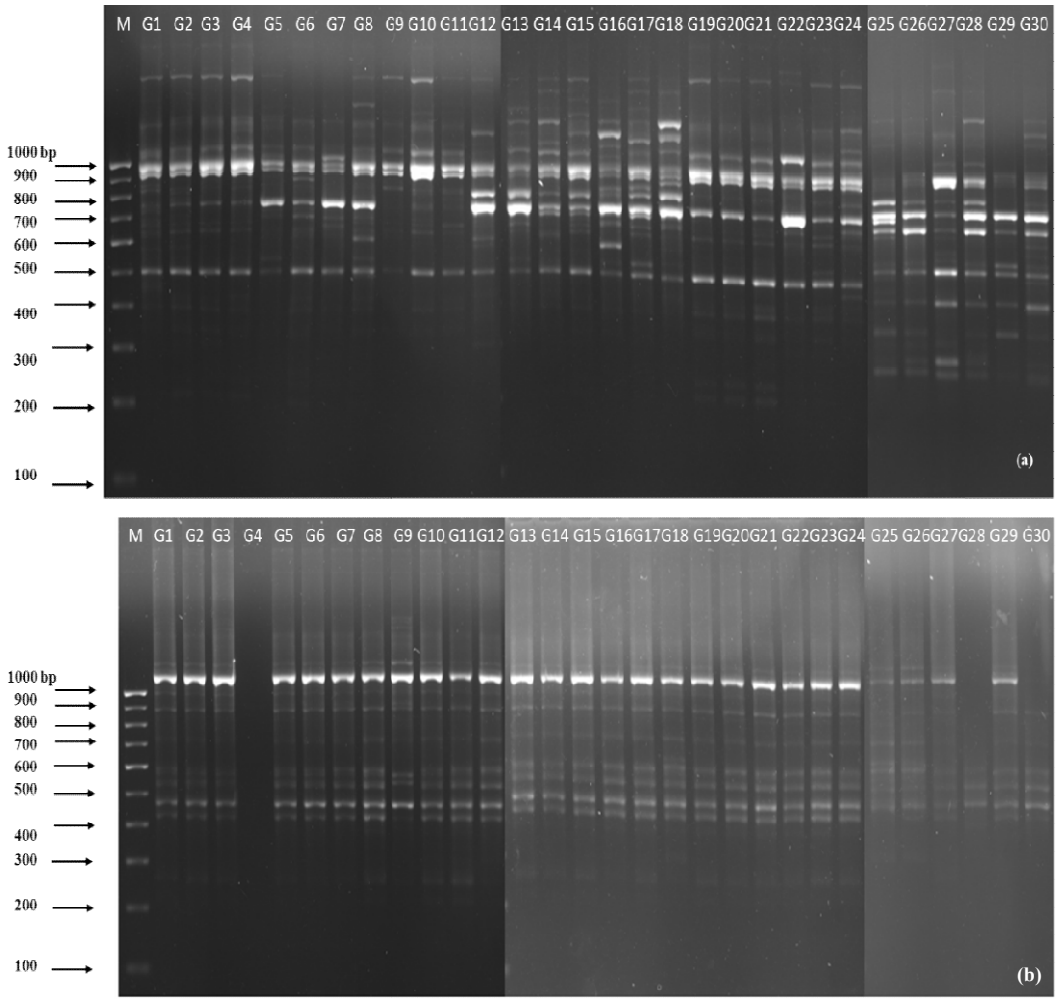

Figure 1.: Typical SCoT (a) and ISSR profile (b) obtained with SCoT-13 and UBC-826 primers respectively-The lanes marked as M are known DNA fragment size markers (100 to 1000 bp). Lanes marked as G1 to G30 represent samples of *Z. officinalis* as denoted in table-1. All amplification products were resolved in 1.5% agarose gels in TBE buffer.

The higher percentage of polymorphism indicates the superiority of SCoT primers over ISSR for genetic diversity analysis. Recently a research group reported the successful use of SCoT markers for the identification of *Fusarium* yellow tolerant/resistant varieties of ginger which were developed through *in-vitro* mutation via gamma irradiation[17]. The size of the amplification products varied in the case of each primer and the range was 0.18–6.0 kb. The extent of polymorphism observed among 30 samples of *Zingiber officinalis* using SCoT and ISSR primers is depicted in Fig. 1.

The average number of amplification products formed by SCoT primers was 14.0; the maximum was 20 from SCoT-13 primer, and the minimum was 7, with SCoT-10 primer. Polymorphic bands were produced in 50.0-92.86% of the amplification products using 13 primers (Table 2). The average number of amplification products formed by the ISSR

primers used was 11.5; the maximum was 15 from the UBC-811 and UBC-826 primers, and the minimum was 7 from the UBC-825. The polymorphic bands produced by the amplification products using 8 primers ranged from 18.88 to 88.89%. Genetic diversity analysis is a base for the selection of good existing germplasm and identifying the gene responsible for a particular trait. SCoT marker was used recently for the authentication of *Fusarium* yellow-resistant lines of ginger (*Zingiber officinale*) developed through gamma radiations[17].Mia et al observed 62.50% polymorphism among eight genotypes from different regions of Bangladesh by implementing the RAPD marker[18]. Due to simplicity and no sequence requirement, RAPD markers were used most extensively to investigate the genetic diversity among ginger genotypes of Meghalaya[19] and subcontinents of India[20]. An overall 92.66% and 94.90% polymorphisms were reported in above mentioned two regions, respectively. Eighty high-recovery dry ginger rhizomes from North East India were studied for genetic diversity through RAPD and ISSR primers. An overall 84.1% and 85.2% polymorphism were reported by respective primers[21]. ISSR and SSR markers were used for the assessment of genetic diversity among ginger cultivars from Odisha and conserved germplasms at the Indian Institute of Spice Research (ISSR), Calicut, Kerela[22].

Cluster analysis

A Neighbour Joining tree after 1000 replicate bootstrap tests of robustness was created to depict the phylogenetic relationship among 30 genotypes of ginger by implementing a pair-wise distance matrix based on the Jaccard coefficient. All 30 genotypes are divided into seven major clusters at a coefficient value of 0.15 (Fig. 2) and the coefficient value ranged from 0.02 to 0.4.

Cluster 1 contained 18 genotypes and was further divided into three subclusters A, B and C which possess 6 genotypes each. Cluster 4, Cluster 5 and Cluster 6 are comprised of three genotypes each. Cluster 2, Cluster 3 and Cluster 7 are solitary containing G12, G8 and G4 genotypes respectively (Table-4). The findings presented in table-1 and fig. 2 indicated strong similarity among genotypes that were collected from a common geographical area and genetic diversity was introduced along with a change in geographical location. Hence, genetic distance is directly proportional to physical distance. Similarly, high genetic diversity was reported earlier by Muda et al.[23] and Jatoi et al.[24] among ginger assessions collected from subcontinents of India and ginger samples collected from Asian regions respectively by using RAPD primers.

Table-4.: Genotypes comprising various clusters as shown in the dendrogram based on UPGMA

Cluster	Subcluster	Genotypes
1	A	G13, G14, G15, G16, 17, G18
	B	G19, G20, G21, G22, G23, G24
	C	G25, G26, G27, G28,G29, G30
2		G12
3		G8
4		G5, G6, G7
5		G9, G10, G11
6		G1, G2, G3
7		G4

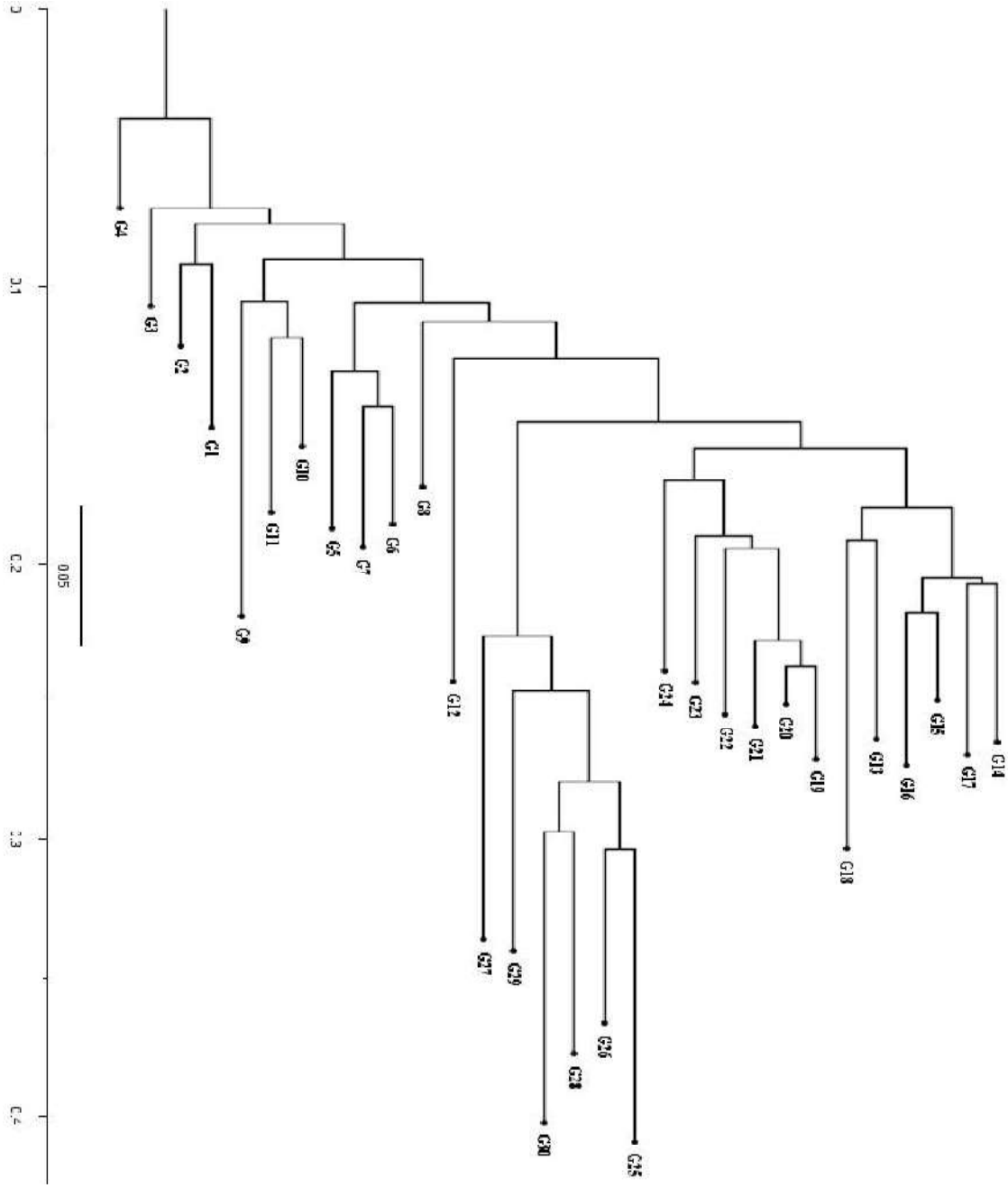

Figure 2.: Neighbour-Joining dendrogram, obtained through the collected dataset of 30 different ginger samples, computed using FreeTree software version 0.9.1.50 and mathematical consensus tree so obtained after 1000 replicates of bootstrap, is viewed and annotated in FigTree version 1.2.2.

*Numbers indicated in the tree are individual sample names as shown in table 1. The branch lengths are based on the distance values computed using Jaccard's coefficient of FreeTree software.

Population genetic analysis

Relationship among populations was determined and a dendrogram was constructed by using Nei's[15] genetic distance unweighted pair group method with arithmetic mean (UPGMA). Results

of population genetic analysis confirmed that members of the DOD population showed the highest genetic diversity while genotypes of population AMI and SAV showed the lowest genetic diversity (Fig. 3). Present investigation revealed that observed and effective number of alleles ranged between 1.0885– 1.6115 and 1.0601– 1.3758, respectively. Nei's gene diversity (h) ranged between 0.0345 and 0.2179 with total diversity at the species level being 0.1769. Shannon's information index (I) ranged between 0.0508 and 0.3254 with an overall diversity of 0.2829 (Table 3). The percentage of polymorphic loci (Pp) was estimated in the range of 8.85– 61.15 with a mean value of 16.87 and a total value of 72.31.

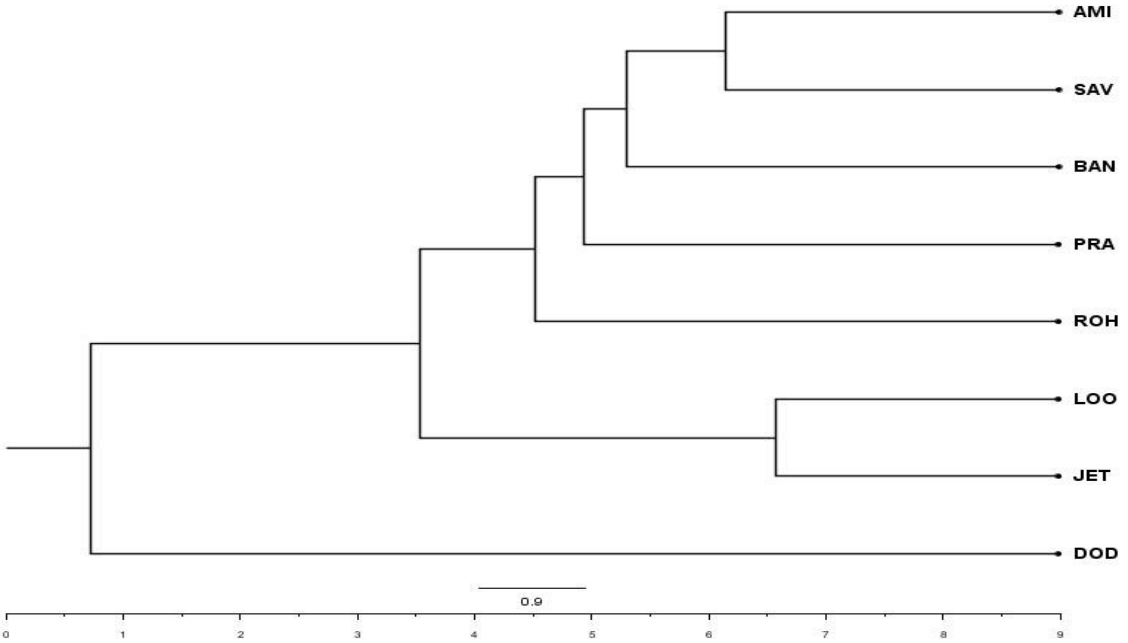

Figure 3.: UPGMA dendrogram showing the relationship among eight populations of *Z. officinalis*collected from two districts of Rajasthan (India).
*Acronym of populations as referred to in table-1.

Conclusion

Being a poorly studied genus, scarce information is available on the molecular characterisation of ginger. This paper first time reports the combined use of SCoT and ISSR markers in the genetic diversity assessment of ginger genotypes collected from different locations in South Rajasthan. A large number of polymorphic bands recommended the combined use of SCoT and ISSR markers could be of significance for the evaluation of genetic diversity in other ginger accessions as well. SCoT primers outperformed ISSR primers based on a higher percentage of polymorphism and a higher number of amplification bands. This study paved the way for future research activities related to genetic diversity assessment, varietal improvement and conservation of potent ginger germplasms from South Rajasthan through extended sampling regimes.

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