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Genetic diversity in henna based on gene targeted SCoT, arbitrarily amplified ISSR and nuclear rDNA multiplicity

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Abstract

Aim : The present study was undertaken to analyze the extent of genetic variability existing among twenty accessions of *Lawsonia inermis*, collected from Rajasthan and Gujarat states of India, using gene targeted SCoT, arbitrarily amplified ISSR and nuclear rDNA markers.

Methodology : Twenty henna accessions, vegetatively established at the Institute were collected from Rajasthan (7) and Gujarat (13). Twenty-six SCoT and twenty ISSR markers generating distinct, unambiguous and scorable fragments were selected, after preliminary screening for assessment of genetic diversity. Data analysis was performed using NTSYS-pc, GenAIEx 6 and POPGENE version 1.31 programs, and dendrograms were generated using unweighted pair group method for arithmetic mean (UPGMA). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using universal primers followed by sequencing and dendrogram generation.

Results : SCoT markers revealed lower values of similarity coefficients ranging from 0.87 - 0.93 compared to 0.93 - 0.98 for ISSR. SCoT markers delineated the *L. inermis* cultivars into three distinct clusters while ISSR markers demarcated them into five clusters.

Interpretation : The Gujarat population of *L. inermis* was richer in genetic diversity than that of Rajasthan. SCoT markers proved better than the ISSR markers for genetic diversity analysis. Substantial variation in ITS-1 region due to SNPs, INDELS and ITS length polymorphism the nucleotide sequences signified its phylogenetic utility in assessing genetic diversity in of *L. inermis*.

Key words: Genetic diversity, ISSR markers, *Lawsonia inermis*, Molecular markers, rDNA, SCoT markers

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Introduction

Lawsonia inermis commonly known as *Henna* or *Mehendi* is an indigenous shrub of warm climatic regions of Africa and Asia (Chaudhary et al., 2010). In India, it is cultivated as a cash crop in Rajasthan (Chand and Jangid, 2007) and Gujarat (Jaimini et al., 2005). *L. inermis* leaves contain 1.7 - 2.4 % 'lawsone'. This dye is extensively used in India during festivals to decorate palms, fingers, fingernails and soles, and for dyeing hair, leather, silk and wool. Leaves, stem bark, flowers, roots and seeds of *L. inermis* are helpful in curing several diseases (Chaudhary et al., 2010). It also possess numerous cosmetic, medicinal and antimicrobial properties (Sharma et al., 2016). *L. inermis* is cultivated vegetatively through cuttings and sexually through seeds. Singh et al. (2005) studied the modes of pollination and reported that maximum fruit set was observed with natural open pollination followed by hand cross pollination while no fruit set was recorded after selfing. The choice of high yielding cultivars is of vital importance for improving the production and productivity of *L. inermis* under different agro-ecological environments. These elite cultivars can be multiplied vegetatively for assured returns. The morpho-physiological characterization of *L. inermis* cultivars has remained inadequate to differentiate diversity within morphological groups. Some groups have also characterized mehendi using phytochemicals i.e., carbohydrates, glycosides, tannins, phenolic compounds, gums, mucilage, naphthoquinones and a number of other constituents (Chaudhary et al., 2010).

Several molecular markers have been used to identify the duplicates or sort the germplasm and to estimate genetic diversity among the plants (Al-Qurainy et al., 2015). Start Codon Targeted (SCoT) markers, designed from the conserved regions flanking the translation initiation codon has emerged as a robust marker technique to reveal genetic variations within and among morphological groups in plants (Chhajer and Kalia, 2017). Recently, SCoT markers have been employed for the assessment of genetic diversity, quantitative trait loci mapping and molecular analyses in plants (Yan et al., 2016; Al-Qurainy et al., 2015). Similarly, Inter-simple sequence repeats (ISSRs), the short inter-tandem repeat DNA sequences exhibiting difference in distinctive genome regions at several loci have also been useful in assessing genetic diversity and evolutionary biology in plants (Kulhari et al., 2015). Both these PCR based, inexpensive marker systems, independent of availability of prior sequence information reveals variations within unique regions of the genome.

Eukaryotic genomes harbor thousands of copies of ribosomal DNA (rDNA) genes arranged in tandem arrays which during the course of evolution exhibits variations and are quite useful for phylogenetic studies. Internal transcribed spacer (ITS) between 18S and 26S rRNA genes, is quite informative region for phylogenetic assessment at inter and intraspecific levels. As compared to highly conserved 5.8S gene, this region varies at a faster rate and has been exploited to resolve and establish inter-species (Mahelkaa et al., 2017) and intra-species

associations (Singh et al., 2017). Variations in nuclear rDNA region at a genus, species and or individual level render it a robust means for estimation of phylogenetic progression and bio-geographical assortment (Andrew and Kohn, 2009). The phylogenetic usefulness of ITS polymorphism has been enumerated in number of crops.

Use of two or more marker systems is known to provide better chances for detection of genetic differences in plants as they usually amplify different regions of the genome (Chhajer et al., 2017). Kulhari et al. (2015) used RAPD and ISSR markers in *Commiphora wightii* while SCoT, ISSR and RAPD were used in potato (Gorji et al., 2011), SCoT and CAAT box-derived polymorphism (CBDP) in jojoba (Heikrujam et al., 2015), EST-SSR and SCoT in Orchard grass (Yan et al., 2016), ISSR, DAMD and SCoT in chickpea (Pakseresht et al., 2013) and SCoT, ISSR and rDNA in *Ziziphus mauritiana* (Singh et al., 2017). The objective of this study was to assess the genetic diversity and relationships among twenty native cultivars of *L. inermis* collected from Rajasthan and Gujarat states using a combination of gene targeted SCoT, arbitrarily amplified ISSR and nuclear rDNA polymorphisms.

Materials and Methods

Fresh leaves of twenty *L. inermis* cultivars vegetatively established at the Institute after collection from Rajasthan (7) and Gujarat (13) were used for genomic DNA isolation using modified CTAB protocol (Doyle and Doyle, 1990). The DNA quality was assessed on 0.8 % agarose gel and quantified at 260 and 280 nm wavelength using Spectrophotometer. Twenty-six SCoT (Collard and Mackill, 2009) and twenty ISSR markers generating distinct, unambiguous and scorable fragments were selected, after preliminary screening, for assessment of genetic diversity among the twenty cultivars of *L. inermis*. DNA amplification reactions were performed in 25 µl volume containing 1x PCR buffer (Sigma Aldrich), 0.2 mM dNTP mix (Thermo Scientific), 1.5 mM MgCl₂, 1 µM SCoT/ISSR primer (Integrated DNA Technologies Inc., India), 50-70 ng of template DNA and 0.6 U Taq DNA polymerase (Sigma Aldrich). PCR was performed in an Eppendorf thermocycler (Mastercycler Nexus GSX1) using the following program: initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 51°C (SCoT)/42°C (ISSR) for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min.

The PCR products were separated on 2% agarose (Amresco, USA) gels containing 1x TAE buffer, stained with ethidium bromide (1.0 µg ml⁻¹ gel; Sigma Aldrich), run under a steady voltage of 110V for 120 min and then photographed under gel documentation system (Alphaimager EC, Protein Simple, California, USA). In all agarose gels, Gene Ruler 100bp plus DNA ladder (Thermo Scientific, EU, Lithuania) was used as size marker. The reproducibility of amplified profiles developed by SCoT and ISSR marker systems was validated by performing the reactions twice. Jaccard's coefficient of similarity of amplified products of SCoT and ISSR were determined by converting it into

binomial matrix (0 or 1) employing NTSYS-pc software. To carry out molecular analyses, twenty *L. inermis* cultivars were grouped into two populations viz., Pop 1: Gujarat and Pop 2: Rajasthan representing two different states from which they were collected. The Analysis of Molecular Variance (AMOVA) and Principal Coordinate Analysis (PCA) were performed using GenAlEx 6 software. Allelic frequencies of SCoT and ISSR markers were used separately to estimate the observed number of alleles (na), effective number of alleles (ne), Nei's gene diversity (h) and Shannon information index (i) using the software POPGENE, version 1.31. Cophenetic coefficient was calculated using Mantel statistic to evaluate the difference in matrices and the dendrograms generated by SCoT and ISSR marker systems using NTSYS programme.

The universal primers ITS-1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS-4 (5' TCC TCC GCT TAT TGATAT GC 3') were used to amplify the internal transcribed spacer (ITS) region of ribosomal DNA, which encompasses 5.8S gene from the genomic DNA of all the twenty cultivars. Each PCR reaction of 50 μ l contained: 1x PCR buffer (Sigma Aldrich), 2.5 mM $MgCl_2$, 160 mM dNTP mix (Thermo Scientific), 50 pmol of each ITS-1 and ITS-4 primers, 1U *Taq* DNA polymerase (Sigma Aldrich) and 200 ng genomic DNA. The PCR reactions were performed using conditions mentioned above with annealing temperature of 50°C. The PCR products were separated on 1.6 % agarose gel prepared in 1x TAE buffer in an electrophoresis run for 100 min at 60 V. The agarose gel was stained with ethidium bromide for 10 min and viewed under UV light in a gel documentation system.

Each PCR product of ITS amplified region was sequenced by Sanger di-deoxy sequencing technology employing ABI prism DNA sequencer using universal forward and reverse primers ITS-1 and ITS-4, respectively. Nucleotide sequences obtained were compared with other highly similar sequences using Basic Local Alignment Search Tool (BLAST) programme of NCBI database. The multiple sequence alignments of ITS region (ITS-1, 5.8S r-RNA gene and ITS-2) were performed using CLUSTAL X (Version 1.8) software to detect single nucleotide polymorphisms. Phenogram based on multiple sequence alignment of rDNA ITS sequences was constructed using Tree View software. All the novel gene sequences were assigned GenBank accession numbers by NCBI, USA. A phylogram was generated from the multiple sequence alignment of twenty henna cultivars along with three reference sequences (KF850586, FJ980380, FM887015) using Clustal X 2.0.11 which depicted bootstrap values using NJ plot software. The phylogenetic relationships among *L. inermis* cultivars and the reference sequences from elsewhere were established on the basis of diversity in Single Nucleotide Polymorphisms (SNPs), insertions/deletions (INDELS), ITS length diversity and/or 5.8S nuclear rDNA regions.

Results and Discussion

Twenty-six SCoT primers detected intra-specific variations in *L. inermis* cultivars and generated a total of 218

distinct DNA fragments ranging in size from 200 to 4000 bp. Out of these, 107 polymorphic amplicons accounted for an average of 45.82% polymorphism with a range of 11.11 % (SCoT-17) to 87.50 % (SCoT-6) polymorphism individually. The number of PCR amplicons varied from 4 (SCoT-2) to 15 (SCoT-3) with an average of 4.16 fragments per primer (Table 1). All the SCoT primers showed high PIC values ranging from 74.4 % (SCoT-2) to 91.4 % (SCoT-3, SCoT-7). The amplified PCR product profile generated with primer SCoT-7 is shown in Fig.1a. Twenty ISSR primers generated a total of 144 amplicons (size range of 175 - 3000 bp) of which 46 were polymorphic and exhibited 31.94 % polymorphism. The number of PCR amplified fragments ranged from 5 (ISSR-18, 19, 28, 29, 33) to 12 (ISSR-40) with an average of 7.2 amplicons per primer (Table 1). PIC values for ISSR markers ranged from 71.1 (ISSR-33) to 91 % (ISSR-40). The gel profile generated using primer ISSR-40 exhibited the maximum polymorphism and PIC values is shown in Fig.1b. Moharana *et al.* (2016) reported an average of 4.5 and 3.3 amplicons per primer using ISSR and RAPD markers, respectively, in *L. inermis* plants regenerated through axillary shoot proliferation. They reported a homogeneous amplification profile for all micro-propagated plants validating their genetic fidelity. PIC analysis indicates discriminating power of a marker and is dependent on the genetic diversity of genotypes selected (Manimekalai and Nagarajan, 2006). The suitability of molecular marker techniques based on the polymorphism detected, range of Jaccard's similarity coefficients and average PIC value were found to be higher in SCoT than ISSR markers.

The UPGMA dendrogram generated using cumulative analysis of 26 SCoT primer matrices through Jaccard's similarity coefficient separated the twenty cultivars in to three distinct clusters with similarity of approximately 0.87 to 0.93% (Fig. 2a). Cluster I included five cultivars M1 (Amirgarh), M2 (Anand), M3 (Dhanduka), M4 (Khedbrahm) and M5 (Kothara) of which M4 was the distinct cultivar. Cluster II contained ten cultivars M8 (SK Nagar), M12 (Ajmer), M13 (Bikaner), M14 (Jobner), M16 (Jadiya), M17 (Pali), M18 (Panchotiya), M19 (Sojat), M20 (Vav), with M15 (Jodhpur) as the most distinct cultivar. The third cluster contained five cultivars M6 (Malan), M7 (Malpur), M10 (Sidhpur), M11 (Vasada), with M9 (Sarotra) as distinct cultivar. The highest genetic similarity was found between cultivars collected from SK Nagar and Jobner followed by cultivars from Jodiya and Vav.

The cluster analysis of 144 ISSR amplicons generated a dendrogram exhibiting 0.93 to 0.98 % similarity among the cultivars (Fig. 2b). Twenty cultivars were delineated in to five distinct phylogenetic clusters - Cluster I included two cultivars M1(Amirgarh) and M18 (Panchotiya); Cluster II grouped six cultivars M4 (Khedbrahm), M6 (Malan), M7 (Malpur), M9 (Sarotra), M10 (Sidhpur) and M11 (Vasada); Cluster III comprised of four cultivars M3 (Dhanduka), M5 (Kothara), M16 (Jadiya) and M20 (Vav); Cluster IV included four cultivars M2 (Anand), M15 (Jodhpur), M17 (Pali) and M19 (Sojat), and cluster V included three cultivars M8 (SK Nagar), M13 (Bikaner) and M14 (Jobner). The cultivar M12 (Ajmer) was recorded as the most distinct out group. The highest genetic similarity was observed between

Fig. 1: Amplified PCR product profile of twenty *Lawsonia inermis* cultivars generated with primers (a) SCoT-7; (b) ISSR-40. M = 100bp plus DNA ladder.

cultivars collected from Bikaner (M13) and Jobner (M14) followed by genotypes from Anand (M2) and Jodhpur (M15). Both SCoT as well as ISSR marker based dendrograms revealed that *L. inermis* cultivars collected from the same state fell under different phylogenetic clusters; cultivars collected from Rajasthan got clustered with those collected from Gujarat and *vice versa*. Boubaya *et al.* (2013) estimated the genetic relationships among 25 germplasm belonging to *L. inermis* using RAPD and ISSR markers, the overall mean genetic similarity ranged from 0.1 to 0.83 based on ISSR and from 0.07 to 0.83 based on RAPD data.

Moderate polymorphism in SCoT (45.82 %) and ISSR (31.94 %) primers, and higher PIC 74.2 - 91.4 % (SCoT) and 71.1 - 91 % (ISSR) with clear delineation of all the twenty cultivars in to well defined distinct clusters revealed moderate genetic variation among the tested cultivars and validated the robustness of the marker systems used. Interestingly, despite very high PIC of all the primers of both SCoT and ISSR markers, genetic diversity was moderate. This is attributed to the fact that higher PIC values depict robustness of individual primer used, whereas, moderate genetic diversity was due to higher similarity coefficient amongst *L. inermis* cultivars. Earlier researchers have also reported similar ranges of genetic diversity in 47 accessions of *Ziziphus nummularia* belonging to cultivated and one wild accession employing ISSR (Singh *et al.*, 2007) and among six populations of Saudi Arabian date palm using SCoT (Al-Qurainy *et al.*, 2015)

markers. Whereas Boubaya *et al.* (2013) reported that overall mean genetic similarity based on ISSR and RAPD data ranged from 0.1 - 0.83 and 0.07-0.83, respectively among twenty five Tunisian germplasm of *L. inermis*. The insight of clustering patterns of both the dendrograms revealed that SCoT markers described *L. inermis* cultivars better than ISSR markers with greater genetic distances. For instance, ISSR markers grouped the twenty cultivars in to five clusters but SCoT markers not only delineated them with higher phylogenetic distances but also grouped them in to three well defined clusters with three distinct cultivars as out groups-M4 (Khedbrahm), M15 (Jodhpur) and M9 (Saroitra). SCoT marker a simple, novel DNA marker technique for generating gene-targeted markers in plants has recently become the marker of choice in genetic diversity studies (Collard and Mackill, 2009; Al-Qurainy *et al.*, 2015; Singh *et al.*, 2017).

Multivariate cluster analysis and principal coordinate analysis (PCA) were used to analyze the data. PCA is an ordination method similar to principal component analysis, except that PCA uses the distance matrix, rather than the values, to plot the axes (Manly, 1994). Principal coordinate analysis (PCA) of two populations using SCoT data exhibited that the contribution of first three eigen factors was 11.29, 9.76 and 9.07 %, respectively, accounting for a total of 30.12 % variability (Fig 3A). On the other hand, contribution of first three eigen factors was 5.97, 4.97 and 4.36 % respectively for the two populations based on ISSR data,

Table 1: Extent of polymorphism among the twenty *Lawsonia inermis* cultivars assessed using SCoT and ISSR primers

Primer code	Primer sequence	GC content (%)	No. of amplicons	No. of polymorphic amplicons	Polymorphism (%)	PIC value (%)
SCoT						
SCoT-2	CAACAATGGCTACCACCC	55.0	4	1	25.00	74.4
SCoT-3	CAACAATGGCTACCACCG	55.0	15	10	66.60	91.4
SCoT-4	CAACAATGGCTACCACCT	50.0	5	3	60.00	77.1
SCoT-5	CAACAATGGCTACCACGA	50.0	14	11	78.57	91.0
SCoT-6	CAACAATGGCTACCACGC	55.6	8	7	87.50	84.6
SCoT-7	CAACAATGGCTACCACGG	55.0	13	11	84.62	91.4
SCoT-8	CAACAATGGCTACCACGT	50.0	10	8	80.00	85.4
SCoT-9	CAACAATGGCTACCAGCA	50.0	8	6	75.00	85.4
SCoT-12	ACGACATGGCGACCAACG	61.1	8	6	75.00	82.7
SCoT-13	ACGACATGGCGACCATCG	61.1	7	5	71.43	78.1
SCoT-14	ACGACATGGCGACCAACG	66.7	8	3	37.50	84.2
SCoT-15	ACGACATGGCGACCGCGA	66.7	6	3	50.00	74.9
SCoT-16	ACCATG GCTACCACCGAC	61.1	10	4	40.00	89.6
SCoT-17	ACCATGGCTACCACCGAG	61.1	9	1	11.11	88.9
SCoT-18	ACCATGGCTACCACCGCC	66.7	6	1	16.66	82.7
SCoT-19	ACCATGGCTACCACCGGC	66.7	6	1	16.67	83.1
SCoT-20	ACCATG GCTACCACCGCG	66.7	5	1	20.00	79.8
SCoT-21	ACGACATGGCGACCCACA	61.1	10	3	30.00	88.4
SCoT-24	CACCATGGCTACCACCAT	55.6	9	3	33.33	88.0
SCoT-25	ACCATGGCTACCACCGGG	66.7	9	3	33.33	88.0
SCoT-26	ACCATGGCTACCACCGTC	61.1	7	2	28.57	84.7
SCoT-28	CCATGGCTACCACCGCCA	66.7	7	1	14.28	85.2
SCoT-29	CCATGGCTACCACCGGCC	72.2	14	5	35.71	91.0
SCoT-30	CCATGGCTACCACCGGCG	72.2	7	3	42.86	82.8
SCoT-33	CCATGGCTACCACCGCAG	66.7	5	2	40.00	79.8
SCoT-35	CATGGCTACCACCGGCC	72.2	8	3	37.50	86.6
ISSR						
ISSR-12	GCAAGACAGACAGACA	50.0	8	1	12.50	86.1
ISSR-18	GGACAGACAGACAGACA	52.9	5	2	40.00	77.9
ISSR-19	GACAGACAGACAGACAG	53.0	5	3	60.00	77.7
ISSR-22	GGGTGGGTGGGGTG	80.0	6	1	16.67	81.1
ISSR-26	GAGAGAGAGAGAGAT	47.1	9	2	22.22	88.6
ISSR-28	CTCTCTCTCTCTCTA	47.1	5	2	40.00	77.8
ISSR-29	CTCTCTCTCTCTCTG	52.9	5	1	20.00	79.7
ISSR-30	GTGTGTGTGTGTGTC	53.0	7	2	28.57	85.7
ISSR-31	GTGTGTGTGTGTGTT	47.1	7	2	28.57	83.6
ISSR-32	TCTCTCTCTCTCTCA	47.1	8	2	25.00	86.3
ISSR-33	TCTCTCTCTCTCTCC	52.9	5	3	60.00	71.1
ISSR-34	TCTCTCTCTCTCTCG	52.9	8	2	25.00	83.9
ISSR-35	ACACACACACACACG	52.9	8	5	62.50	84.9
ISSR-39	CACACACACACACAGC	56.0	9	5	55.56	87.3
ISSR-40	ACACACACACACACCA	50.0	12	2	16.67	91.0
ISSR-41	ACACACACACACACTG	50.0	7	3	42.86	81.8
ISSR-45	AGAGAGAGAGAGAGAT	44.4	8	1	12.50	87.0
ISSR-46	AGAGAGAGAGAGAGAA	44.4	9	4	44.44	86.7
ISSR-47	AGAGAGAGAGAGAGTA	44.0	7	1	14.29	84.1
ISSR-49	ACTGACTGACTGACTG	50.0	6	2	33.33	77.2

Fig. 2: UPGMA dendrogram of twenty *Lawsonia inermis* cultivars generated using cumulative analysis of (a) 26 SCoT and (b) 20 ISSR primer matrices through Jaccard's Similarity Coefficient.

these eigen factors explained a total variability of 15.3 % (Fig 3B). Wider distribution of population 1 (Gujarat) across the principal coordinates was observed as compared to population 2 (Rajasthan). The mean values of two populations together for Nei's gene diversity (h) was 0.1603 and 0.0870, and Shannon information index (i) was 0.2396 and 0.1326 for SCoT and ISSR markers, respectively. The results indicated that the genetic

diversity of Gujarat population was higher than that of Rajasthan population. Greater contribution of eigen factors, higher Nei's gene diversity and Shannon information index employing SCoT markers, as compared to that of ISSR indicate that SCoT markers have more resolving power than ISSR in revealing genetic variability in *L. inermis* germplasm. G_{st} values imply differentiation over multiple loci and the rate of gene flow signify

Table 2: Summary of nuclear rDNA region of twenty *Lawsonia inermis* cultivars

Cultivar no.	Location (Cultivar)	District	State	GenBank accession number	ITS-1 (bp)	5.8 S (bp)	ITS-2 (bp)	Total (bp)
M1	Amirgarh	Banaskantha	Gujarat	KY515299	234	164	222	620
M 2	Anand	Kheda	Gujarat	KY515300	232	164	222	618
M 3	Dhanduka	Gandhinagar	Gujarat	KY515301	232	164	222	618
M 4	Khedbrahm	Sabarkantha	Gujarat	KY515302	232	164	222	618
M 5	Kothara	Kachchh	Gujarat	KY515303	232	164	222	618
M 6	Malan	Banaskantha	Gujarat	KY515304	232	164	222	618
M 7	Malpur	Sabarkantha	Gujarat	KY515305	232	164	222	618
M 8	SK Nagar	Banaskantha	Gujarat	KY515306	232	164	222	618
M 9	Sarotra	Banaskantha	Gujarat	KY515307	232	164	222	618
M 10	Sidhpur	Patan	Gujarat	KY515308	232	164	222	618
M 11	Vasada	Deesa	Gujarat	KY515309	232	164	222	618
M 12	Ajmer	Ajmer	Rajasthan	KY515310	232	164	222	618
M 13	Bikaner	Bikaner	Rajasthan	KY515311	232	164	222	618
M 14	Jobner	Jaipur	Rajasthan	KY515312	232	164	222	618
M 15	Jodhpur	Jodhpur	Rajasthan	KY515313	232	164	222	618
M 16	Jadiya	Banaskantha	Gujarat	KY515314	232	164	222	618
M 17	Pali	Pali	Rajasthan	KY515315	232	164	222	618
M 18	Panchotiya	Pali	Rajasthan	KY515316	232	164	223	619
M 19	Sojat	Pali	Rajasthan	KY515317	232	164	222	618
M 20	Vav	Banaskantha	Gujarat	KY515318	232	164	222	618

Fig. 3: Principal Coordinates Analysis (PCA) of two populations of *Lawsonia inermis* using (a) SCoT and (b) ISSR primer matrix data exhibiting contribution of eigen factors.

transfer of different alleles and or genes across populations. In present study, higher G_{ST} values indicated better differentiation over multiple loci and greater gene flow gave an insight of higher rate of migration of genes between *L. inermis* populations. Both SCoT and ISSR markers revealed higher values of total gene diversity, gene diversity within each population, coefficient of gene differentiation and level of gene flow indicating robustness of the markers used. SCoT markers revealed comparatively higher values for gene diversity in sub-divided populations as compared to ISSR.

In the present study, a positive correlation between SCoT and ISSR marker systems validate that Cophenetic coefficient was acceptable in both the molecular marker systems. The correlation coefficient (r) between SCoT and ISSR (significant $P > 0.05$) was 0.10 with approximate t value of 1.15. Principally, the values of mantel test correlation determine the fitness of any clustering. In present study a positive correlation between the SCoT and ISSR marker systems validated that Cophenetic coefficient was acceptable in both the molecular marker systems indicating good fit for clustering. Pakseresht *et al.* (2013) carried out mantel test among ISSR, DAMD and SCoT markers and

reported that SCoT and DAMD markers were helpful in fingerprinting of chickpea genotypes. Recently, Singh *et al.* (2017) also reported positive correlation between SCoT and ISSR markers with correlation coefficient (r) of 0.24 (significance $P > 0.05$) with an approximate t value of 3.57 in Indian jujube cultivars. To further analyze gene differentiation among the two populations, Nei's unbiased measure of genetic distance was employed. The results exhibited a genetic distance of 0.0216 (SCoT) and 0.0142 (ISSR) between the two populations while genetic distance showed comparable values of 0.9786 (SCoT) and 0.9859 (ISSR) using two marker systems. Summary of analysis of molecular variance (AMOVA) showed that the proportion of variations within populations was 90 (ISSR) to 91 % (SCoT), and between two populations was 10 (ISSR) to 9 % (SCoT). Cross pollinated behavior of species very well explains the existence of more genetic variability at intra-specific level. Nei's analysis of gene diversity in subdivided populations showed values of total gene diversity (H_t) and diversity within each population (H_s) at 0.1589 and 0.1457, using SCoT markers these values were almost half for ISSR markers being 0.0880 and 0.0793, respectively. Whitlock and McCauley (1999) apprehended that the changes in allelic frequencies are due to

Fig. 4: Phylogram of multiple sequence aligned rDNA region of twenty *Lawsonia inermis* cultivars along with three reference sequences generated using clustal X 2.0.22 and NJ plot software.

migration in or out of a given population often result in variations in genes in the established gene pool. Nei's unbiased measure of genetic distance analysis of SCoT and ISSR markers indicated richer genetic diversity in Gujarat population as compared to that of Rajasthan. Nevertheless, SCoT marker system proved better than ISSR between the two populations of *L. inermis* in terms of unbiased measure of genetic distances.

All the twenty cultivars of *L. inermis* generated a single prominent amplified product, approximately 675 bp long, of 5.8S gene region when subjected to PCR with ITS-1 and ITS-4 universal primers. The ITS amplified region of each cultivar contained few base pairs of 5' end of 18S gene; complete sequence of ITS-1, 5.8S gene and ITS-2; and a few initial base pairs of 28S gene. A total of 20 novel gene sequences revealing variation on account of SNPs, INDELS and ITS length polymorphism were submitted to NCBI database, assigned GenBank accession numbers and have been released in public domain for reference and comparisons (Table 2). The 5.8S rDNA region recorded a uniform length of 164bp in all the 20 cultivars, demonstrating highly conserved nature of the 5.8S rDNA. The ITS-2 region also showed a uniform length of 222bp in all the cultivars except M18 collected from Panchotiya (223bp). Similarly, ITS-1 region also recorded a uniform length of 232bp except the genotype M1 collected from Amirgarh (234 bp). Therefore, the total length of complete 5.8S gene region diverged in size from 618 - 620 bp. Variations in base pair lengths in ITS-1 and ITS-2 regions signifies the phylogenetic utility of ITS region specifically in assessing genetic diversity and relationships of *L. inermis* germplasm. The ITS region length polymorphism by way of SNPs, INDELS and ITS have also been reported to be of phylogenetic significance in determining genetic diversity both in crop plants *Cyamopsis tetragonoloba* (Pathak et al., 2010), *Vigna radiata* (Raturi et al., 2012); and tree species *Punica granatum* (Singh et al., 2013), *Cycas* (Xiao et al., 2010) and *Ziziphus mauritiana* (Singh et al., 2017).

BLAST search of all the cultivars exhibited 98-99% identity with the best aligned GenBank reference sequences. The multiple sequence alignment of all the cultivars of *L. inermis* along with three reference sequences (KF850586, FJ980380, FM887015) downloaded from NCBI GenBank generated a phylogram using clustal X 2.0.22 and NJ plot (Fig 4). The cultivars M2 (Anand) and M7 (Malpur) showed maximum proximity with the reference sequences whereas cultivars M1 (Amirgarh), M4 (Khedbrahm), M9 (Saroitra) and M18 (Panchotiya) were recorded as the most distinct out groups. This considerable intra-specific genetic diversity was due to SNPs, INDELS and ITS length polymorphism in the nucleotide sequences and significant boot strap values (Hillis and Bull, 1993). The clustering patterns of SCoT, ISSR and ITS phylograms revealed no consistency in grouping of *L. inermis* cultivars based on their collection from different geographical regions indicating that *L. inermis* has wide genetic diversity and distribution across agro-climatic zones. Low correlation between geographic distribution and genetic distances has also been reported in other plant species

Cyamopsis tetragonoloba (Pathak et al., 2010) and *Ziziphus mauritiana* (Singh et al., 2017). Boubaya et al. (2013) also reported lack of correlation between geographic locations and genetic diversity while assessing twenty-five Tunisian germplasm of *L. inermis* using ISSR and RAPD marker systems. The clusters based on ISSR and RAPD marker data were independent of their geographical origin.

This is the first study documenting genetic diversity among Indian henna cultivars, collected from major henna growing states, using gene targeted SCoT, arbitrarily amplified ISSR and nuclear rDNA markers. This study revealed existence of more variability within populations rather than among populations. Therefore, identification and maintenance of superior lawsone producing cultivars will help henna producers to earn more economic benefits and will also assist in formulating efficient conservation strategies for this important species.

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