International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 6 | Issue 3| JUL-SEP | 2016 | 128-137
Original Research Article – Biological Sciences

ISSR MARKERS FOR DETERMINATION OF GENETIC DIVERSITY IN SPILANTHES SPECIES - MEDICINAL HERB FROM PENINSULAR INDIA

[1&2]Lavanya Devi K., [1]Anuradha Sane and [2]M.B. Shivanna

[1]Division of Plant Genetic Resources, Indian Institute of Horticultural Research, Bengaluru, Karnataka, India- 560089

[2]Department of Applied Botany, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shivamogga, Karnataka, India- 577451.

***Corresponding Author Email: lavanyakotekar@gmail.com**

ABSTRACT

Inter-simple sequence repeat (ISSR) analysis was carried out to determine the genetic diversity of six species of Spilanthes in peninsular India. Eighteen primers with good banding pattern were selected for the study based on their reproducibility. The results of PCR based ISSR analysis revealed 96.51% polymorphism and 0.19 to 0.35 polymorphic information content. The marker index (4.57 to 8.63) and observed heterozygosity (H_o) (0.2, UBC 854) to (0.54, UBC 856) with an average of 0.36, expected heterozygosity (H_e) (0.18 UBC 854 to 0.35 UBC 853) with an average of 0.26 were documented for all Spilanthes accessions. The Shannon's diversity index ranged from 0.84 (UBC 857) to 1.46 (UBC 853). The average resolving power was 19.87 per ISSR primer. The highest RP value of 25.69 was observed with ISSR primer UBC 836 and the lowest of 15.96 with UBC 873. The phylogenetic analysis indicated that individuals were morphologically distinct species and formed individual clusters. The principal coordinate analysis indicated correlation with similarities and the geographic distances among the species. The Mantel test proved the correlation of molecular matrix with morphological and geographic distance matrix among the accessions. The prevalence of diversity in Spilanthes species in peninsular India is demonstrated for the first time using ISSR markers.

KEY WORDS

Spilanthes, ISSR, PIC, phylogeny, Mantel test.

INTRODUCTION

Spilanthes Jacq. (~ spp. Family: Asteraceae, tribe: Heliantheae) is a specious group distributed both in the New and Old World tropics, as well. Amongst six species of Spilanthes *viz., S. paniculata* Wallich *ex* DC. and *S. calva* (DC.) R.K. Jansen are native to India, while, *S. uliginosa* (Swartz) Cassini, *S. ciliata* (Kunth) Cassini and *S. radicans* (Jacquin) R.K. Jansen. are immigrant species (Shivarajan and Mathew, 1984). *S. oleracea* Linnaeus R.K. Jansen, a cultivated species introduced from Brazil (Hooker, 1872). Ray headed species include *S. calva*, *S. ciliata,* S. *paniculata*. and *S. uliginosa*, while the discoid headed include *S. oleracea* and *S. radicans*. Most of the species find importance in traditional medicines and alkamides extracted from them are effective in curing toothache and throat infections; it is also used as local anesthetic and digestive tonic (Ramsewak et al. 1999). It is reported to possess anti-inflammatory, antiseptic, analgesic, antioxidant and cytotoxic properties (Jondiko et al. 1986; Saritha et al. 2002; Rai et al. 2004; Wu et al. 2008; Prachayasittikul et al. 2009).

The molecular markers have been used in various activities like plant breeding, conservation of genetic resources, disease resistance, gene mapping, linkage studies and genetic diversity studies. The most common markers involved are random amplified polymorphic DNA (RAPD, Welsh and McClelland,

1990; Williams et al. 1990), amplified fragment length polymorphism (AFLP, Vos et al. 1995), restriction fragment length polymorphism (RFLP, Botstein et al. 1980), diversity array technology (DART, Wenzl et al. 2004), sequence-tagged sites (STS, Bradshaw et al. 1994), single nucleotide polymorphism (SNP, Wang et al. 1998), single simple repeat (SSR, Tautz, 1989; Weber and May, 1989), and inter-simple single repeat (ISSR, Zietkiewicz et al. 1994). The ISSR markers were developed from the need to explore microsatellite repeats without prior knowledge of DNA sequences (Lagercrantz et al. 1993). The technique is based on the amplification of DNA segments between two microsatellite repeated regions (Zietkiewicz et al. 1994). Hence, the ISSR is a simple, quick and an efficient technique that produces amplified products in the range of 200–2000 base pairs. The ISSR marker (dominant) system is based on the use of 15-20 bp primers which are complimentary to SSR (microsatellite) sequences found throughout the eukaryotic genomes. The microsatellite repeats target multiple genomic loci to amplify their ISSR sequences of different sizes. In addition, this could be used to confirm the presence and evaluate the distribution of certain microsatellite repeats within the genome (Zietkiewicz et al. 1994). This is useful in identifying individual specific differences, inter-species genomic finger-printing, genetic diversity, phylogenetic inferences, gene tagging, genome mapping and evolutionary biology (Godwin et al. 1997; Reddy et al. 2002; Panda et al. 2003; Bornet and Branchard, 2004; Zhang et al. 2013). The ISSR markers are widely applied in taxonomic studies due to high variation of banding sequences at the population and interspecific level (Vanderpoorten et al. 2003; Dogan et al. 2007) as well as in studies investigating genetic diversity at the species level (Gunnarsson et al. 2005; Liu et al. 2007; Szczecinska et al. 2009). Although morphological variations in *Spilanthes* species are documented, there is no molecular evidence on genetic diversity of these species. In the present study, the molecular characterization of *Spilanthes* species was attempted by using ISSR markers to determine the inter-specific and intra-specific variations occurring in *Spilanthes* distributed in the peninsular India and correlation among molecular, morphological and geographical distance of the accessions.

MATERIALS AND METHODS

Collection of plant material

In the present study, six species of *Spilanthes viz., S. calva, S. ciliata, S. oleracea, S. paniculata, S. radicans* and *S. uliginosa occurring* in the peninsular India were selected for their characterization by using the ISSR technique. Plants collected from various locations (Fig.1). were grown in a shade house at Field Gene Bank of Division of Plant Genetic Resources, Indian Institute of Horticultural Research (IIHR), Bangalore, India. Details of collection locations from peninsular India are detailed in Table A1(supplementary material).

Protocol for optimization of plant DNA isolation and PCR-ISSR analysis

The genomic DNA was extracted principally by the Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The plant parts exhibited the presence of phenols that interfered in obtaining pure DNA sample, hence modified CTAB method was employed to obtain clear creamish DNA. The purity checking was carried out using the UV spectrophotometer where the optical density ratio was optimum (OD260:280 around 1.8-2.0). The gel electrophoresis also exhibited clear DNA bands. A preliminary optimization of DNA isolation and 7-8 PCR ISSR analyses showed that the amplification could be carried out as described previously (Lavanya et al. 2014). In this study, 75 ISSR primers from University of British Columbia (UBC) were screened initially and 18 primers that yielded amplification products for all six species were selected for PCR ISSR analysis. The details of the primers and sequences are detailed in Table A2 (supplementary material).

Characteristics of amplified bands from ISSR markers

Each ISSR band was identified as an independent locus and distinct, reproducible bands were considered for scoring. The scoring was carried out manually by observing for the presence of ISSR bands (presence =1 or absence =0) to determine polymorphism in each locus. The amplified product of each primer was determined for the total number of amplified bands; number of polymorphic bands (bands not amplified in all accessions) and number of monomorphic bands (bands present in all

accessions), maximum number of bands, minimum number of bands and average bands.

Analysis of ISSR markers

In order to determine the suitability of ISSR markers for evaluation of genetic profiles, the performance of markers was measured using parameters such as percentage polymorphism (PP), polymorphic information content (PIC), Nei's genetic distance, observed heterozygosity (Ho), expected heterozygosity (He), marker index (MI), resolving power (RP) and principal coordinate analysis (PCA).

The PIC refers to the value of a marker for detecting polymorphism within a population. The PIC depended on the number of detectable alleles and the distribution of their frequency (Boostein *et al.*, 1980, Anderson et al. 1993). Hence, the PIC of each primer was calculated using the average PIC value from all loci of each primer. The MI was calculated as described (Varshney et al. 2005).

MI = EMR × PIC

Effective multiplex ratio (EMR) = n × β

where n is the average number of bands amplified by accession to a specific system marker (multiplex ratio) and β is estimated from the number of polymorphic loci (np).

The number of non-polymorphic loci (nnp), β = np/ (np + nnp).

The RP of each primer was calculated (Prevost and Wilkinson, 1999) as- RP = Σ Ib

where Ib represents the informative fragments. The Ib can be represented on a scale of 0-1 by the following formula: Ib = 1 - (2 × (0.5 - p))

where p is the proportion of accessions containing the band.

The genetic diversity of ISSR marker was evaluated by calculating Shannon diversity Index

Shannon diversity index (H) was calculated (Lewontin, 1972) as- H= Σpilog2pi

where pi is the frequency of a given band for each population.

To find the relationship between genetic composition and geographic distribution of accessions, genetic composition and morphological data matrix and all the three were tested for correlation using the partial Mantel test (Mantel 1967; Mantel and Valand 1970). The Euclidean distance matrix (D) was computed and converted to a similarity(S) matrix by formula S= 1 - D. A Nei's genetic similarity matrix (Nei and Li, 1979) was also computed for the genetic data. Using the geographical coordinates of the collection localities, a geographic distance matrix was computed and converted into a similarity matrix. All matrices were tested for pair-wise correlation ($p<0.05$), using Mantel's non-parametric test (Mantel, 1967).

The principal coordinate analysis (PCO) was performed. The Nei's genetic distance matrix was subjected to phylogenetic analysis (Parsimony method, Paup Verison 4.0) to assess genetic relationships among accessions. All above statistical analyses were determined by the program NTSYS-pc 2.11.

RESULTS AND DISCUSSION

ISSR scoring and data analysis

The selected primers (18) generated a total of 2215 bands from 30 accessions, amongst which 2137 bands were polymorphic (96.50%). The maximum amplification was observed due to primer 855 (163 fragments) and minimum observed due to primer 844 (38 fragments). Each primer caused various levels of polymorphism to differentiate the species and 100% polymorphism was observed in primers- UBC 841, 842, 844, 845, 846, 847, 852, 853, 854, 855, 858, 860 and 866 (Table 1). The band length ranged from 134 to 1100. The fragment numbers were 34-76 for 844, 854 and 857; 78-120 for 860, 866 and 858; 121-140 fragments for 843, 845, 842 and 852; 141-165 fragments for 841, 853, 846, 847, 856, 840 and 855 (Figure 1). The results indicated that ISSR markers could be used effectively to analyze the genetic diversity in *Spilanthes.* Although species of *Spilanthes* viz., *S. calva, S. ciliata, S. oleracea, S. paniculata, S. radicans* and *S. uliginosa* in peninsular India could be identified morphologically (Lavanya et al. in press), there is a published report analysis of genetic diversity of *Chrysanthemum* varieties at the varietal and species levels, which was determined successfully by ISSR markers (Arup et al. 2013).

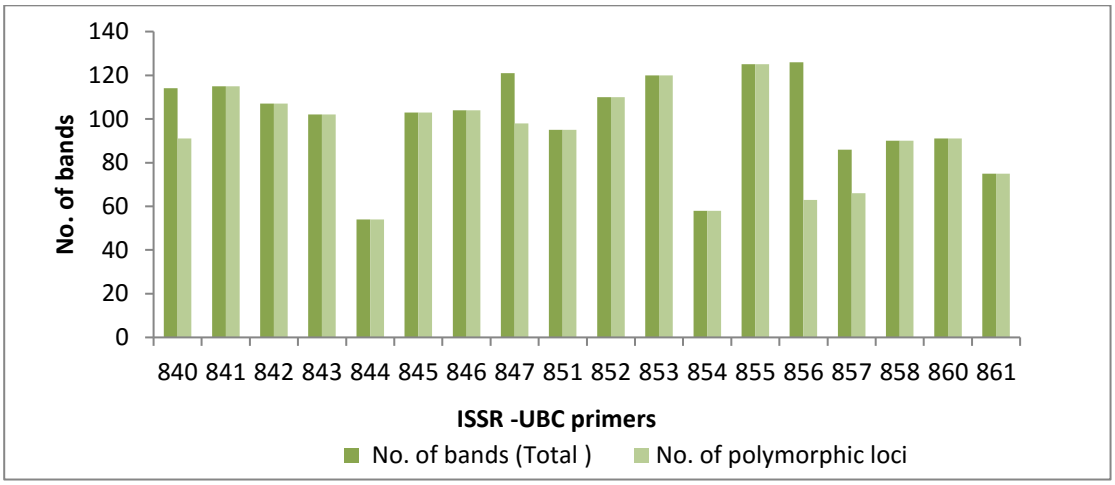

Figure 1: The number of bands and polymorphic loci yielded for 30 accessions of *Spilanthes* spp. using UBC ISSR primers.

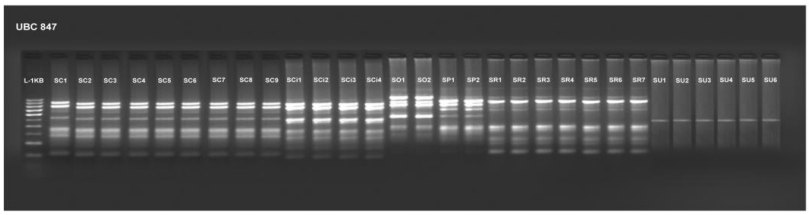

Figure 2: ISSR finger-prints generated using primer UBC 856 from 30 accessions of *Spilanthes* collected from peninsular India

Table 1: Amplified products of UBC ISSR markers and their characteristics

SN	UBC ISSR Primer	TNB	NPB	PP	MXNB	MNNB	AVG B
1.	840	9	8	88.88	9	2	4.95
2.	841	9	9	100	8	2	5
3.	842	9	9	100	7	1	4.6
4.	843	8	7	87.5	6	1	4.43
5.	844	7	7	100	4	1	2.34
6.	845	9	9	100	9	1	4.47
7.	846	8	8	100	8	1	4.42
8.	847	10	9	100	8	5	4.26
9.	851	8	7	87.5	6	3	4.13
10.	852	11	11	100	8	1	4.78
11.	853	11	9	100	8	2	5.21
12.	854	9	9	100	8	2	2.52
13.	855	9	9	100	7	4	5.43
14.	856	8	7	87.5	8	4	2.73
15.	857	7	6	85.7	5	1	2.86
16.	858	8	8	100	6	3	3.91
17.	860	9	9	100	8	2	3.95
18.	866	10	10	100	10	2	3.26

Data screening is based on 30 accessions of *Spilanthes* collected from peninsular India.
TNB= total number of bands; NPB= number of polymorphic bands; PP=percentage polymorphism; MXNB= maximum number of bands; MMNB= minimum number of bands; AVGB- average number of bands per primer).

Table 2: Genetic diversity of *Spilanthes* accessions and parameters studied using 18 UBC ISSR primers.

Sl. No.	UBC ISSR Primer	Ho	He	FR	DI	MI	PIC	RP	SDI
1.	840	0.46	0.29	0.91	0.73	7.33	0.26	5.4	1.16
2.	841	0.39	0.28	1	0.65	6.53	0.24	5.13	1.21
3.	842	0.37	0.28	1	0.64	7.07	0.27	5.6	1.23
4.	843	0.37	0.21	0.91	0.77	8.47	0.20	3.8	0.88
5.	844	0.22	0.19	1	0.55	6	0.15	4.47	0.92
6.	845	0.38	0.29	1	0.46	4.57	0.30	7.67	1.41
7.	846	0.38	0.29	1	0.8	9.6	0.27	6.47	1.23
8.	847	0.41	0.33	1	0.56	6.2	0.30	7.93	1.53
9.	851	0.37	0.26	1	0.59	6.5	0.24	4.2	1.02
10.	852	0.41	0.33	1	0.63	6.97	0.30	7	1.4
11.	853	0.44	0.35	1	0.59	7.03	0.33	7.47	1.46
12.	854	0.2	0.18	1	0.62	6.83	0.16	4.8	1.06
13.	855	0.54	0.29	1	0.62	6.83	0.27	2.33	0.87
14.	856	0.45	0.32	1	0.78	8.63	0.30	5.6	1.19
15.	857	0.23	0.18	1	0.63	5.7	0.15	3.6	0.84
16.	858	0.36	0.27	1	0.63	7.6	0.25	5.2	1.1
17.	860	0.35	0.23	0.92	0.61	7.3	0.21	6.07	1.19
18.	861	0.27	0.24	1	0.54	4.88	0.22	5.33	1.1

Data screening is based on 30 accessions of *Spilanthes* collected from peninsular India.
Ho=observed heterozygosity; He=expected heterozygosity; Fr=frequency of polymorphic bands; DI=diversity index; MI=marker index; PIC=polymorphic information content; RP=resolving power; SDI=Shannon diversity index for 18 UBC ISSR primers.

The PIC analysis was carried out to determine the efficiency of each ISSR marker to express polymorphic loci in *Spilanthes*. The calculated PIC values for ISSR markers ranged from 0.15 to 0.33. The highest mean PIC value of 0.33 expressed by primer UBC 853 indicated that this primer was the most polymorphic. The primer UBC 857, which yielded the lowest mean PIC value of 0.15, was the least polymorphic. The MI ranged from 4.57 for UBC 845 to 8.63 for UBC 856 which indicated that the latter primer was the most efficient in detecting polymorphism.

As far as the genetic diversity parameters are concerned, observed heterozygosity (Ho) ranged from 0.2 (UBC 854) to 0.54 (UBC 856) with an average of 0.36, whereas the expected heterozygosity (He, genetic diversity) ranged from 0.18 (UBC 854) to 0.35 (UBC 853) with an average of 0.26 for *Spilanthes* accessions. The Shannon diversity indices (SDI) ranged from 0.84 (UBC 857) to 1.46 (UBC 853). The resolving power (RP) is a parameter that indicated the discriminatory potential of primers chosen. While the average RP was 19.87 per ISSR primer, the highest RP value was observed with the ISSR primer 836 (25.69) and the lowest with the ISSR primer 873 (15.96) (Table 2).

Polymorphism in a given population occurs due to the existence of genetic variants which is represented by the number of alleles at a locus and their frequency of distribution in a population. In the present study, the percentage of polymorphism due to UBC ISSR markers was high as revealed by the

differentiation of species. There are several comparative studies with RAPD showing high polymorphism than the ISSR (Shaffie et al. 2011).

The technique of ISSR has been successful in determining the difference among polyploids and diploids (Reddy et al. 2002). The individual polyploids have resulted following hybridization. The genus *Spilanthes* is self-pollinated but random crossing could have resulted in the formation of hybrids (Jansen, 1980). The change in morphological characteristics such as radiate to discoid heads and the reduction in the number of floral parts support the possibility of hybridization of self-pollinated randomly crossing species of *Spilanthes* on opined by Jansen (1980). The ISSR markers could be employed for these investigation to differentiate the taxa status at species and varietal level.

The Mantel test for ISSR data matrix with geographical data for collection locations of species, ISSR similarity matrix and morphological similarity matrix (Lavanya et al. 2014 in press) indicated a positive correlation. Correlation of three matrices by partial Mantel test was positive as well, indicating the good correlation among three data sets.

Correlation test conducted for each morphological character with each primer and *vice-versa* and with geographic distance of accessions with the genetic similarity matrix and *vice-versa*. Among morphological characters' flower head diameter and leaf lamina length exhibited good correlation (r=0.3673 and r=0.2103). The morphological characters like stipule size, leaf lamina width, peduncle length exhibited moderate correlation, but it was not significant (p>0.05). Whereas correlation of each primer with morphological similarity matrix when performed, exhibited high correlation for UBC 840 and low for 861 (r=0.6928 and r=0.2774), correlation value (r>0.6) was achieved for UBC 840, 842, 847, 851 and 860. The correlation values (r<0.3) achieved for UBC 854 and 861 and all values were significant (p <0.05). The partial Mantel test correlated for three similarity matrices, a positive correlation (r=0.648) was achieved and was significant (p=0.002). The correlation among the genetic and geographical distances showed r=0.2207 with p value of 0.4404; on the other hand, the genetic similarity matrix with morphological similarity matrix correlation was positive (r=0.6455 and p=0.001).

Principal coordinate analysis (PCA) The related species were clearly separated by principal coordinate analysis (PCA). The grouping of samples in principal coordinate analysis was according to the morphological grouping of species of *Spilanthes* and accessions were segregated within the genus according to geographical variations and similarities within the species (Fig 3).

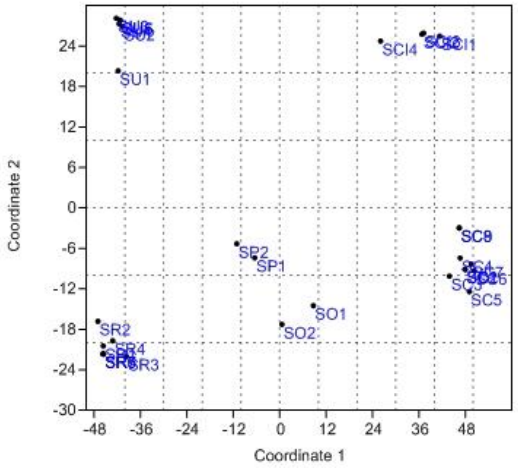

Figure 3. Two-dimensional representation of principal coordinate analysis of genetic relationships of Spilanthes spp. inferred from similarity matrix using the Jaccard's index by UBC ISSR markers. *S. calva*:SC1-SC9, *S. ciliata*: SCI1-SCI4, *S. oleracea*: SO1, SO2, S. *paniculata*: SP1, SP2, *S. radicans*:SR1-SR7, *S. uliginosa*: SU1-SU6.

Cluster analysis

Based on the genetic distances, the phylogenetic tree was constructed to determine the relation of *Spilanthes* accessions at the interspecific, intra-specific levels and within the species of *Spilanthes* accessions. The species were differentiated clearly in the tree, with each species forming an individual sub-cluster. Accordingly, species *S. uliginosa* and *S. radicans* were closely related, while S. *paniculata* was closely related to this sub-cluster, whereas *S. oleracea* and *S. paniculata* were clustered together. The cluster of *S. oleracea, S. paniculata* and *S. radicans* and the cluster of S. *calva* and *S. ciliata* were connected to third sub-cluster. Two accessions of *S. calva* were separated from the species individual cluster. The clusters clearly differentiated the species and within the species the distance among

accessions were correlated to the geographical distances. The correlation between the genetic data and geographical distances and correlation with morphological similarity matrix and all three revealed by the Mantel test, was confirmed by the PCO analysis (Fig 3) and clearly so by the Parsimony dendrogram (Fig 4).

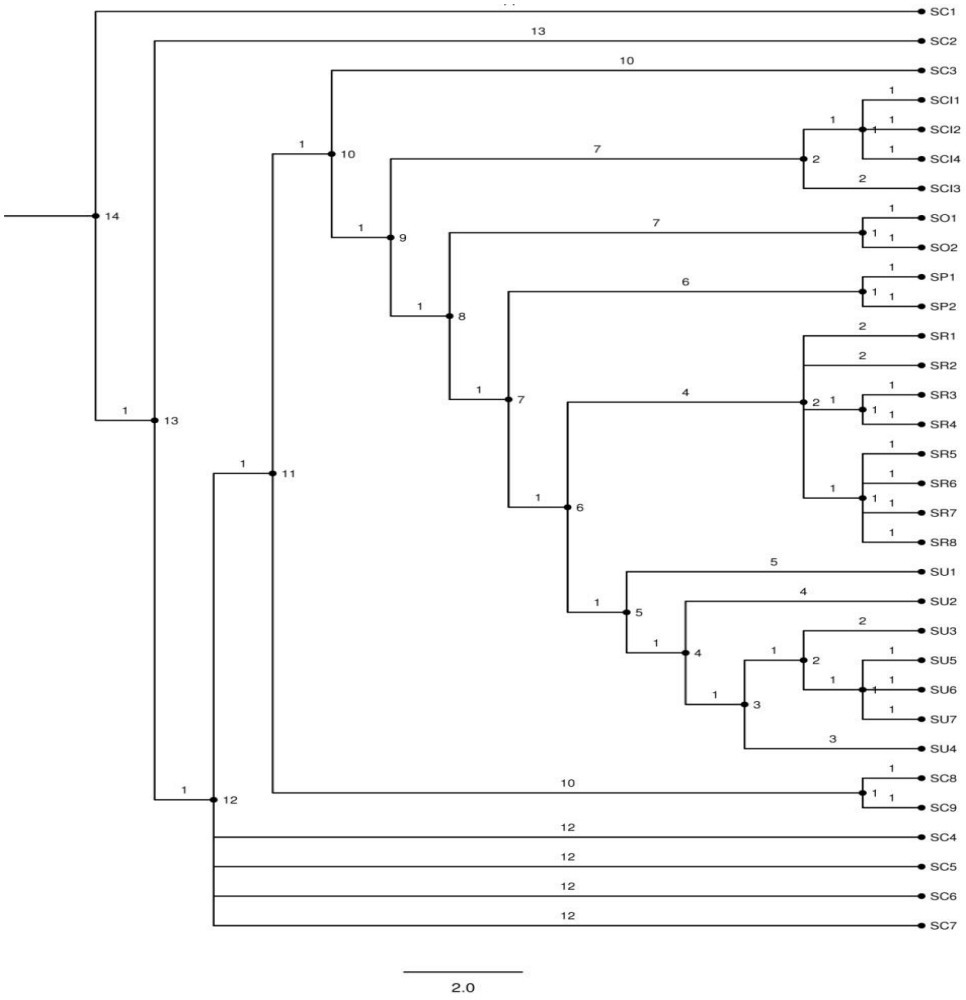

Figure 4. Phylogeny of *Spilanthes* accessions generated by PAUP on the similarity matrix obtained using Nei's genetic distance based on ISSR data (Nei, 1978).

CONCLUSION

In the present study the markers were technique of ISSR used for successful differentiation the species of *Spilanthes* occurring in peninsular India. Further the existence of polyploids and their relationship with diploid individuals during the course of evolution could be studied. The genus *Spilanthes* is known to be closely related to genus *Acmella* (Richard, 1807) and is characterized by the presence of discoid heads and pappus of awns. Taxonomic studies by Cassini in 1822 that focused on the characterization of two genera *Spilanthes* and *Acmella* concluded that *Acmella* differs from *Spilanthes* only in having radiate heads and hence these two genera cannot be separated reliably by differences of the pappus. Since then, this genus has been traditionally divided into two sections, *Spilanthes* and *Acmella* Rich (Candolle 1836; Moore 1907). More recently morphological and chromosomal studies suggest that *Acmella* and *Spilanthes* should be considered as two distinct genera and the features used to separate the taxa are described as follows (Jansen 1980; 1985). In this context a detailed study of both the taxa *Spilanthes* and *Acmella* is need of the hour. The species distributed in Peninsular India morphologically are in accordance with the *Acmella* species described by Jansen (1981, 1985). In this context, the molecular study (ISSR) of these species is a foundation for

studies of these taxa and their revision in Peninsular India.

ACKNOWLEDGEMENT

The authors sincerely thank The Director, Indian Institute of Horticultural Research Bangalore, Karnataka, India for providing facilities for carrying out the present research. The authors sincerely thank all those who helped in the field collections.

REFERENCES

1. Anderson JA, Churchill GA, Autrique JE, Tanksley S, Sorrells ME (1993). Optimizing parental selection for genetic linkage maps. *Genome*. 36:181–186.
2. Angeles JGC, Laurena AC, Mendoza EMT (2005). Extraction of genomic DNA from the lipid, polysaccharide and polyphenol rich coconut (*Cocos nucifera* L.). *Plant Mol. Biol. Rep* 23:297–298.
3. Arup KM, Atanu D, Laxmikant A, Siddhartha KP, Prathap CP (2013). Studies on genetic diversity in elite varieties of *Chrysanthemum* using RAPD and ISSR markers. Indian Journal of Biotechnology. 12:161–169.
4. Bornet B, Branchard M (2004). Use of ISSR fingerprints to detect microsatellites and genetic diversity in several related Brassica *taxa* and *Arabidopsis thaliana*. *Hereditas* 140:246–248. DOI:10.1111/j.1601-5223.2004.01737.
5. Botstein D, White RL, Skolnick M, Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32:314–331.
6. Bradshaw HDJ, Villar M, Watson BD, Otto KG, Stewart S (1994). Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS and RAPD markers. *Theor. Appl. Gene.* 89:167–178.
7. Dogan B, Duran A & Hakki EE (2007). Phylogenetic analysis of *Jurinea* (Asteraceae) species from Turkey based on ISSR amplification. *Ann. Bot. Fenn.* 44:353–358.
8. Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus* 12:13–15.
9. Geiduschek EP, Herskovits TT (1961). Non aqueous solutions of DNA. reversible and irreversible denaturation in methanol. Arch Biochem. *Biophys*. 95:114–129.
10. Godwin ID, Aitken EAB, Smith LW (1997). Application of Inter Simple Sequence Repeat (ISSR) markers to plant genetics. *Electrophoresis*. 18:1524–1528. DOI: 10.1002/elps.1150180906.
11. Grant V, Plant *speciation.* 2nd edn. (1981) Columbia University Press, New York.
12. Gupta M, Chyi YS, Romero-Severson J, Owen JL (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. *Theor. Appl Genet.* 89:998–1006.
13. Hundsdoerfer AK, Kitching IJ, Wink M (2005). The phylogeny of Hyles *euphorbiae* complex (Lepidoptera: Sphingidae): Molecular evidence for sequence data and ISSR-PCR fingerprints. *Org. Divers Evol.* 5:173-198. DOI: 10.1016/j.ode.2004.11.012.
14. Jansen R K, Systematics of *Spilanthes* (Compositae :Heliantheae) (1981). *Syst. Bot*. 6:231–257.
15. Jansen RK (1985) The systematics of *Acmella* (Asteraceae - Heliantheae). *Syst. Bot. Monogr.,* 8: 1-115.
16. Jenny KA, Daniel J, Crawford, Arnoldo S, Mark EM (2006). The utility of automated analysis of Inter-Simple Sequence Repeat (ISSR) loci for resolving relationships in the Canary Island species of *Tolpis* (Asteraceae). *Am. J. of Bot.* 93:1154–1162.
17. Jondiko IJO (1986). A mosquito larvicide in *Spilanthes mauritiana*. *Phytochemistry*. 25:2289–2290
18. Joshi SP, Gupta VS, Agarwal RK, Ranjekar PK, Brar DS (2000). Genetic diversity and phylogenetic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theor. Appl. Genet*. 100:1311–1320.
19. Khare CP (2007). *Indian medicinal plants: An illustrated dictionary.* 621.
20. Kozyrenko MM, Gontcharova SB, Gontcharoy AA (2011). Analysis of the genetic structure of *Rhodiola rosea* (Crassulaceae) using inter-simple sequence repeat (ISSR) polymorphisms. Flora. 206:691–696.
21. Kunsiri C, Grubbs AE, Randall L, Small AE, Edward ES (2009). Evidence for multiple, autoploid origins of agamospermous populations in *Eupatorium sessilifolium* (Asteraceae). *Plant Systematics and Evolution*. 279:151–161. DOI 10.1007/s00606-009-0155-y.
22. Lagercrantz U, Ellegren H, Andersson L (1993). The abundance of various polymorphic

microsatellite motifs differs between plant and vertebrates. *Nucleic Acids Res.* 21:1111–1115.

23. Lavanya DK, Anuradha S, Shivanna MB, Ganeshan S (2014). Optimization of DNA isolation and PCR ISSR of species of *Spilanthes* from peninsular India. *Inter. J. Sci. Res.*3(9):1088–1091.
24. Lewontin RC (1972). The apportionment of human diversity. Evolutionary Biology. 6:381–398.
25. Liu XQ, Grruru RW, Chen LQ (2007) Genetic variation in the endangered fern *Adiantum reniformae* var. *sinensis* (Adiantaceae) in China. *Ann. Bot. Fenn.* 44:25–32.
26. Nei M (1972). Genetic distance between populations. *Am. Nat.* 106:283–292.
27. Panda S, Martin JP, Aguinagalde I (2003). Chloroplast and nuclear DNA studies in a few members of the *Brassica oleracea* L. group using PCR-RFLP and ISSR-PCR markers: A population genetic analysis. *Theor. Appl. Genet*. 106:1122–1128. DOI: 10.1007/s00122-002-1134–7.
28. Pasakinskiene I, Griffiths CM, Bettany AJE, Paplauskiene V, Humphreys MW (2000). Anchored simple-sequence repeats as primers to generate species-specific DNA markers in *Lolium* and *Festuca* grasses. *Theor. Appl. Genet*. 100:384–390.
29. Powell W, Morgante M, Andre C, Hanafey M, Voges J, Tingey S, Rafalski A (1996). A comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol. Breed*. 2: 225-230.
30. Prevost A, Wilkinson MJ (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Gen.* 98 107–112.
31. Qian W, Ge S, Hong DY (2001). Genetic variation within and among populations of a wild rice *Oryza granulate* from China detected by RAPD and ISSR. *Theor. Appl. Genet.* 102:440–449. DOI: 10.1007/s001220051665.
32. Rai MK, Varma A, Pandey AK (2004). Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. *Mycoses.* 47:479–481.
33. Ramsewak RS, Erickson AJ, Nair MG (1999). Bioactive N isobutylamides from the flower buds of *Spilanthes acmella*. *Phytochemistry*. 51:729–732.
34. Reddy MP, Sarla N, Siddiq EA (2002). Inter Simple Sequence Repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*. 128:9–17. DOI: 10.1023/A:1020691618797.
35. Rees WA, Yager TD, Korte J, Hippel VPH (1993). Betaine can eliminate the base pair composition dependence of DNA melting. *Biochemistry* 32:137–44.
36. Salimath SS, de Oliveira AC, Godwin ID, Bennetzen JL (1995). Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. *Genome*, *38*:757–763.
37. Saritha KV, Prakash E, Ramamurthy MN (2002). Micropropagation of *Spilanthes acmella* Murr. *Biol. Plantarum*. 45: 581–584.
38. Szczecinska M, Sawicki J, Wasqwicz K, Holdynski CZ (2009). Genetic variation of the relict and endangered population of *Chamaedaphne calyculata* (Ericaceae) in Poland. *Dendrobiology.* 62:23–33.
39. Shafie M, Shafie B, Hasan SMZ, Abdullah MZ, Ramisah MS (2011). RAPD and ISSR markers for comparative analysis of genetic diversity in wormwood capillary (*Artemisia capillaris*) from Negeri Sembilan, Malaysia. *Journal of Medicinal Plants Research.* 5:4426–4437.
40. Shivarajan VV, Mathew P (1984). Notes on three immigrant species of *Spilanthes* Jacq. Asteraceae in India and the identity of the common "Toothache Plant". *Anc. Sci. of Life.* 3:169–73.
41. Stebbins (1980). Review of ‘'Plant Evolution through amphiploidy and autopolyploidy. *Ecology.* 26: 420–421.
42. Tautz D (1989). Hypervariability of simple sequences as a general source of polymorphic DNA markers. *Nucleic Acids Res*, 17:6463–6471.
43. Tsumura Y, Ohba K, Strauss S (1996). Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). *Theor. Appl. Genet.* 92:40–45.
44. Vanderpoorten A, Hedenas L, Jacquemart AL (2003). Differentiation in DNA fingerprinting and morphology among species of *Pleurocarpus* moss genus, *Rhytidiadelphus* (Hylocomiaceae). *Taxon*. 52:229–236.
45. Varshney RK, Graner A, Sorrells ME (2005). Genic microsatellite markers in plants: features and applications. *Trends Biotechnol.* 23:48–55.
46. Prachayasittikul V, Prachayasittikul S, Somsak R, Prachayasittikul V (2009). High therapeutic potential of *Spilanthes acmella*: A review. *EXCLI Journal* 12:291–312. – ISSN 1611-2156.
47. Virk PS, Zhu J, Newbury HJ, Bryan GJ, Jackson MT, Ford-Lloyd BV (2000). Effectiveness of different

classes of molecular marker for classifying and revealing variation in rice (Oryza sativa) germplasm. *Euphytica*. 112:275–284.

48. Vos P, Rogers R, Bleeker M, Reijans M, Van-de-Lee T, Hornes M, Fritjers A, Pot J, Peleman J, Kuipe M, Zabeau M (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23:407–4414.
49. Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J (1998). Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science*. 280:1077–1082.
50. Weber May, Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44 (1989) 388–396.
51. Welsh J & McClelland M, Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res*. 18 (1990) 7213–7218.
52. Wenzl P, Li H, Carling J, Zhou M, Raman H, Paul E, Hearnden P, Maier C, Xia L, Caig V, Ovesna J, Cakir M, Poulsen D, Wang J, Raman R, Smith K, Muehlbauer G, Chalmers K, Kleinhofs A, Huttner E, Kilian A (2006). A high-density consensus map of barley linking DArT markers to SSR, RFLP and STS loci and agricultural traits. BMC *Genomics.* 7:206. doi: 10.1186/1471-2164-7-206.
53. Williams JKF, Kubelik AR, Livak KG, Rafalki JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res*. 18:6531–6535.
54. Wolff K, Zietkiewicz E, Hofstra H (1995). Identification of chrysanthemum cultivars and stability of DNA fingerprint patterns. Theor. Appl. Genet. 91:439–447.
55. Wu LC, Fan NC, Lin MH, Chu IR, Huang SJ, Hu CY, Han SY (2008). Anti-inflammatory effect of spilanthol from *Spilanthes acmella* on murine macrophage by down-regulating LPS-induced inflammatory mediators. J. Agric. Food Chem. 56:2341–2349.
56. Zhang L, Zhang HG, Xf L (2013). Analysis of genetic diversity in *Larix gmelinii* (Pinaceae) with RAPD and ISSR markers. Genet. Mol. Res., 12:196–207. PMID: 23408406.
57. Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by Simple Sequence Repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176–183. DOI:10.1006/geno.1994.1151.

***Corresponding Author:**
Lavanya Devi K*
Email: lavanyakotekar@gmail.com