© 2018 IJSRST | Volume 4 | Issue 5| Print ISSN: 2395-6011 | Online ISSN: 2395-602X
Themed Section: Scienceand Technology

Genetic Diversity and Similarity Studies in different Accessions of *Decalepis hamiltonii* by Random Amplified Polymorphic DNA (RAPD) Analysis

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ABSTRACT

DecalepishamiltoniiWight &Arnis a valuable endemic and endangered medicinal shrub. The species is commonly called as maredukommulu or barresugandhi or maradugaddalu or makaliberu belonging to the family Asclepediaceae. Its roots have been used in Ayurveda, the ancient Indian traditional systems of medicine to stimulate appetite, skin diseases, diaphoretic, hemorrhoids, rheumatism, bronchitis, diaphoretic, somatic and antiviral and as a general tonic. It is also useful as a blood purifier, preservative, diarrhea, respiratory disorders and urinary disorders. The present paper deals with Genetic diversity and similarity studies in different accessions of Decalepishamiltonii by Random Amplified Polymorphic DNA analysis. The study was taken up with 15 RAPD Primes, distance matrix (dendrogram) using Unweighted Pair Group Method with Arithmetic averages (UPGMA) or neighbour-joining, were calculated using Jaccard's similarity. The maximum genetic similarity among the collected species was sixty five percentages which was calculated by Jaccard's coefficient. These results could be very useful to study and manage the genetic resources of an important medicinal plant.

Key words: Decalepishamiltonii, Roots, Accessions, RAPD, Dendrogram, UPGMA, Genetic similarity

I. INTRODUCTION

DecalepishamiltoniiWight &Arn. The species is endemic and endangered to peninsular India geographical distribution of D. hamiltoni is in southern India and rare in evergreen forests of Western Ghats and commonly called as maredukommulu or barresugandhi or maradugaddalu or makaliberu belonging to the family Asclepediaceae (Anonymous, 2003; Raju and Ramana, 2009). It has been recorded in the dry and moist deciduous forests of Karnataka, Andhra Pradesh and Tamil Nadu. Its roots have been used in Ayurveda, the ancient Indian traditional systems of medicine to stimulate appetite, skin diseases, diaphoretic, hemorrhoids, rheumatism, asthma, bronchitis, diaphoretic, somatic and antiviral and as a general tonic (Nayor et al., 1978). It is also useful as a blood purifier, preservative, diarrhea, respiratory disorders, fever, bronchitis, asthma, eye diseases, urinary disorders, loss of appetite, burning sensation and rheumatism and especially for epileptic fits in children and as a source of bio insecticide for stored food grains (George et al., 1999). Earlier studies have shown that roots contains aldehyde, inositols, amyrins and lupeols (Murti et al., 1940) as well as volatile compounds such as 2-hydroxy- 4-methoxy benzaldehyde, vanillin, 2-phenylethyl alcohol, benzaldehyde and others (Nagarajan et al., 2001).

The plant has a use in many medicinal preparations and due to this there is a heavy demand for it. As the whole plant is uprooted from its natural habitat for the use of the herbal drug industry its numbers are decreasing drastically in the natural population. To meet the huge demand for its supply, there is a need to develop a specific technology for production of D. hamiltoniiin a large scale. The maintenance of genetic purity is a limitation for large scale cultivation.

Decalepis is one of the most important medicinal and economical properties or related information of all the species of Decalepis over its range of distribution, current status and the role of biotechnology in the conservation of this important genus. The roots of D. hamiltonii are little bitter and then sweet. It is so characteristic with a familiar lingering after taste and smell of vanillin, the substance that is in Vanilla planifolia, an orchid used in ice-creams, chocolates, drinks etc. Although vanillin has been synthesized since 1874 natural source of this flavoring are still in demand and the roots of Decalepis species can be used as substitute for vanillin (Sharma, 2014).

Genetic variation has implications for the conservation the species level. These Molecular markers can characterize plants with greater precision than the biochemical parameters (Polanco et al., 2002). Among these, Random Amplified Polymorphic DNA (RAPD) markers are efficient to assess genetic variation and have been used extensively to evaluate natural genetic diversity in plant populations (Nybom et al., 2000; Li. J.M et al., 2006). Due to its procedural simplicity, the use of RAPD as molecular marker for taxonomic and systematic analyses of plants, as well as in plant breeding and the study of genetic relationships has considerably increased (Ranade et al., 2001). It is informative and fast for the assessment of population structure, genetic diversity and phylogenetic analysis. The RAPD markers are efficient to assess genetic variation and have been used extensively to evaluate natural genetic diversity in plant populations (Nybom et al., 2000). The present study on Decalepishamiltonii has been taken up to study its genetic diversity.

II. MATERIAL AND METHODS

Molecular analysis of the germplasm for study of genetic diversity by RAPD:

The plant material used in study consisted of Decalepishamiltonii were collected from different geographical regions of Telangana and Andhra Pradesh states in India like (1) Sri Venkateshwara University, Tirupathi, Andhra Pradesh (DH-1), (2) Herbal Garden Agriculture University from, Rajendranagar, Ranga Reddy District, Telangana (DH-2), (3) Pragathi Green Nursery Proddutur, Ranga Reddy District (DH-3), (4) Andhra Pradesh Medicinal Plants Board (APMPB), Chilukur Hyderabad (DH-4), (5) Central Institute of Medicinal and Aromatic Plants from Boduppal, Hyderabad District, Telangana (DH-5) and (6) Botanical garden, Department of Botany, Osmania University, (DH-6). were raised in Botanical garden, Department of Botany, Osmania University. After acclimatization these were used in the genetic diversity studies through Randomly Amplified Polymorphic DNA (RAPD) analysis (Table-1).

Table 1. Particulars accessions of Decalepis hamiltonii collected different places

S. NO	Genotype	Genotype ID
1	Sri Venkateshwara University, Tirupathi, Andhra Pradesh	DH-1
2	Herbal Garden Agriculture University from Rajendranagar, Ranga Reddy District, Telangana	DH-2
3	Pragathi Green Nursery Proddutur	DH-3
4	Andhra Pradesh Medicinal Plants Board, Chilukur, Hyderabad (APMPB)	DH-4
5	Central Institute of Medicinal and Aromatic Plants from Boduppal, Hyderabad District, Telangana	DH-5
6	Botanical garden, Department of Botany, Osmania University, Hyderabad	DH-6

The RAPD analysis was taken up from the DNA isolated from young leaves of the accessions. The technique comprised the isolation of DNA, qualitative and quantitative analysis of DNA by electrophoresis, followed by the polymerase chain reaction (PCR) by employing random primers. The DNA isolation was carried out according to the protocol of Doyle and Doyle (1990).

The leaves were ground in the extraction buffer and after homogenization treated with RNase and precipitated. It was washed with 70 % ethanol and dried. The DNA was dissolved in 100 ml of Tris EDTA. The purity was checked by electrophoresis and by spectrophotometric analysis. The concentration of DNA was adjusted to 50 ng/µl. A total of 15 random primers were used in amplification (OPC-1, OPC2-, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, OPC-8, OPC-9, OPC-10, OPC-11, OPC-12, OPC-13, OPC-14 and OPC-15.

The PCR conditions followed were:

Chemicals and reagents, PCR Reaction set up:

The PCR amplification reactions of the RAPD method were conducted in 25 µl volume reaction mixture containing of 10 × PCR Buffer, 25 Mm $Mgcl_2$, Template DNA 50 ng, 10 Mm d NTP'S, Random primers (10 PM / µl), Taq DNA polymerase, Nuclase free water (15µl).

PCR amplification was carried out in Thermocycler with the following conditions:

Initial denaturation was done at 94°C for 5 minutes, followed by denaturation for 40 cycles at 94° C for 1 minutes, Annealing for 1 minute at 35° C followed by 2 minutes for extension at 72°C and final extensions at 72° C for 10 minutes.

RAPD data analysis:

RAPD-PCR amplified fragments were scored as 1 for present and 0 for absent. The binomial data generated was used to estimate level of polymorphisms by dividing the polymer bands by the total number of scored bands. The RAPD Data was analyzed using CERVUS (3.03 Version). Dendrogram was constructed by UPGMA (Unweighted Pair Group Method with Arithmetic mean) method to measure the resulting phenotypic groups. Pairwise comparision between accessions based on the proportion of shared bands produced by the primers used, were calculated using Jaccard's similarity.

III. RESULTS AND DISCUSSION

In the present study an attempt was made to characterize the six accessions of Decalepishamiltonii collected from different places like (1) Sri Venkateshwara University, Tirupathi, Andhra Pradesh (DH-1), (2) Herbal Garden Agriculture University from, Rajendranagar, Ranga Reddy District, Telangana (DH-2), (3) Pragathi Green Nursery Proddutur, Ranga Reddy District (DH-3), (4) Andhra Pradesh Medicinal Plants Board (APMPB), Chilukur, Hyderabad (DH-4), (5) Central Institute of Medicinal and Aromatic Plants from Boduppal, Hyderabad District, Telangana (DH-5) and (6) Botanical garden, Department of Botany, Osmania University, (DH-6). for genetic diversity studies. Very limited or no work has been reported so far for the genetic diversity studies in Decalepishamiltonii. Hence the present study was carried out to provide important information regarding the genetic material for selection and for conservation of this very important medicinal plant.

DNA isolation and purity:

CTAB method was used for isolation of genomic DNA with little modification. Good quality DNA was isolated from tender leaves of six accessions of Decalepishamiltonii. The pure DNA was determined by running the samples on 1% agarose gel and

compared with lambda (λ) DNA marker. Amount of DNA was determined from the concentration of DNA in the eluate. It was measured by absorbance at 260 nm/ 280 nm. DNA purity was determined by calculating the ratio of absorbance at 260 nm to absorbance of 280 nm (Fig:-1). The isolated DNA was of good quality as it showing a reading between 1.7 - 2.14 is showing the purity of DNA. The concentration of purified DNA was varying from 0.6µg /µl.

Primer selection:

Total of fifteen random primers were used for DNA amplification. Among these, six primers (OPC-1, OPC-4, OPC-6, OPC-9, OPC-11 and OPC-13) were showed good amplification. Nine primers (OPC-2, OPC-3, OPC-5, OPC-7, OPC-8 and OPC-14) were showed medium amplification.

PCR amplification:

The PCR amplification was performed in thermo cycler. The three primers (OPC-10, OPC-12 and OPC-15) were amplified under following PCR conditions. The initial denaturation was done at 94°C for 5 min followed by 1 cycle, denaturation at 94°C for 1 min and extension was at 72°C for 2 min followed annealing at 35°C for 1min by 35 cycles and a final extension was at 72°C for 10 min. The nine primers (OPC-1, OPC-4, OPC-6, OPC-9, OPC-11 and OPC-13) were amplified with following PCR conditions. The initial denaturation was done at 94°C for 5 min followed by 1 cycle, denaturation at 94°C for 1 min and extension was at 72°C for 2 min followed annealing at 34°C for 1min by 35 cycles and a final extension was at 72°C for 10 min.

Polymorphism:

In the present study, RAPD primers generated good number of polymorphic bands and polymorphism was clearly distributed.Good quality genetic profiling was obtained with using fifteen random primers for six accessions collected from different places. In the present study, a total number of 75 bands appeared in which 43 bands were polymorphic bands and 32 monomorphic bands. The maximum numbers of bands 5 to 9 were obtained with four primers (OPC-1 5-bands, OPC-13 6-bands, OPC-6 7-bands, OPC-9 8-bands, OPB-4 8-bands and OPC-11 9-bands). Among the 9 bands produced by OPC-11 primer, 8 were polymorphic and 1 was monomorphic band, OPC-9 shown 8 polymorphic and 1 monomorphic bands, OPC-4 shown 8 polymorphic, OPC-9 primer 8 polymorphic bands (Fig: - 2, 3 and 4). The highest percentage (25%) of polymorphism was shown in three primers (OPC-11, OPC-1, and OPC-13). 20% of polymorphism was shown in OPC-9 followed by 16.6% of polymorphism in OPC-6.The lowest percentage (12.5%) of polymorphism was shown in OPC-4 (Table-2).

Polymorphic information content (PIC):

The Decalepishamiltonii polymorphism level of markers is compared by polymorphic information content. The PIC is recorded as the mean which was calculated from frequency of polymorphic bands among all the genotypes. The PIC values were ranged between 0.094-0.682. The highest PIC value was shown in OPC-11 as 0.682 followed by 0.672 in OPC-1, 0.278 in OPC-13, 0.222 in OPC-10 and 0.127 in OPC-6. The lowest PIC value (0.094) was recorded in OPC-4 (Table-2).

Cluster analysis

The genetic similarity matrix were prepared on the basis of all amplified products of six accessions of Decalepishamiltonii with fifteen primers, with the help of dendrogram which was generated by UPGMA cluster analysis of Jaccard's similarity coefficient. It showed two clusters with a wide range of variability among six accessions. Cluster A consists of four accessions (Sample-DH-1, DH-4, DH-6 and DH-2) and

cluster B consists of 2 accessions (Sample-DH-3 and DH-5) (Figure 5).

Genetic similarity:

The genetic similarity among the six accessions of Decalepishamiltonii has shown the range between 20%- 65%. The maximum similarity (65%) was observed among the accessions Sample-5 and sample-4. This was calculated by Jaccard's coefficient (Table-3). The genetic distance between the six accessions was ranged from 0.7534 to 0.9393. The genetic distance was calculated by Jaccard's distance coefficient (1.00000).

Table 2. List of amplified primers used for RAPD analysis, unamplified primers, total no. of bands. Polymorphic and monomorphic bands and polymorphism information content of six accessions of Decalepishamiltonii

PRIMER	SEQUENCE	TM (°C)	GC CONTENT (%)	No. of Bands	Band Range	PIC	Polymorphism
OPC-1	TTCGAGCCAG	41.8	70	5	<500-3000	0.672	Polymorphic
OPC-2	GTGAGGCGTC	34.4	60	6	<500-<3000		Monomorphic
OPC-3	GGGGGTCTTT	31.3	60	7	500->3000		Monomorphic
OPC-4	CCGCATCTAC	32.5	60	8	<500-3000	0.094	Polymorphic
OPC-5	GATGACCGCC	31.2	60	5	500-<3000		Monomorphic
OPC-6	GAACGGACTC	32.7	60	7	<500->3000	0.127	Polymorphic
OPC-7	GTCCCGACGA	31.8	60	5	<500-1500		Monomorphic
OPC-8	TGGACCGGTG	33.1	60	4	<500-1000		Monomorphic
OPC-9	CTCACCGTCC	39.9	70	8	<500-<3000	0.222	Polymorphic
OPC-10	TGTCTGGGTG	36.5	60	Not clear	500-1000		
OPC-11	AAAGCTGCGG	35.5	60	9	500-2000	0.682	Polymorphic
OPC-12	TGTCATCCCC	33	60	Not clear	0		
OPC-13	AAGCCTCGTC	33.4	60	6	500-<3000	0.278	Polymorphic
OPC-14	TGCGTGCTTG	34.8	70	5	500-1500		Monomorphic
OPC-15	GACGGATCAG	39	70	Not clear	500-2000		

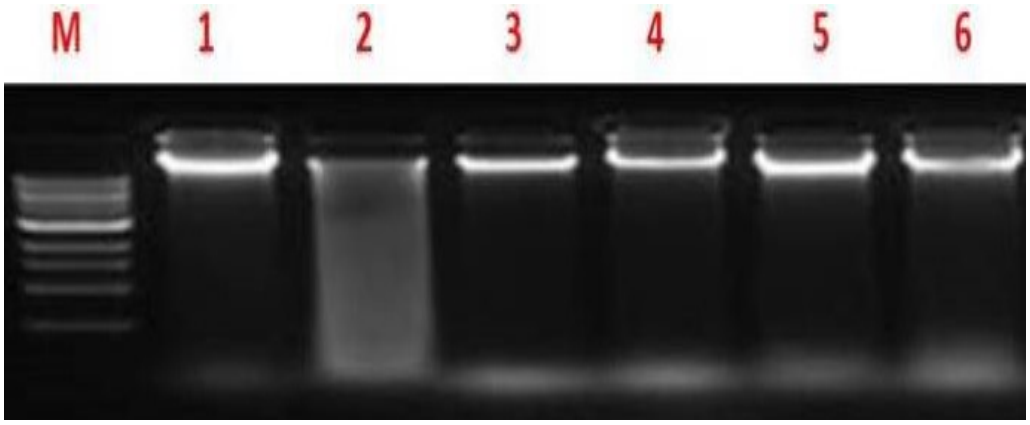


Figure 1. Extracted Genomic DNA from Six accessions of Decalepishamiltonii

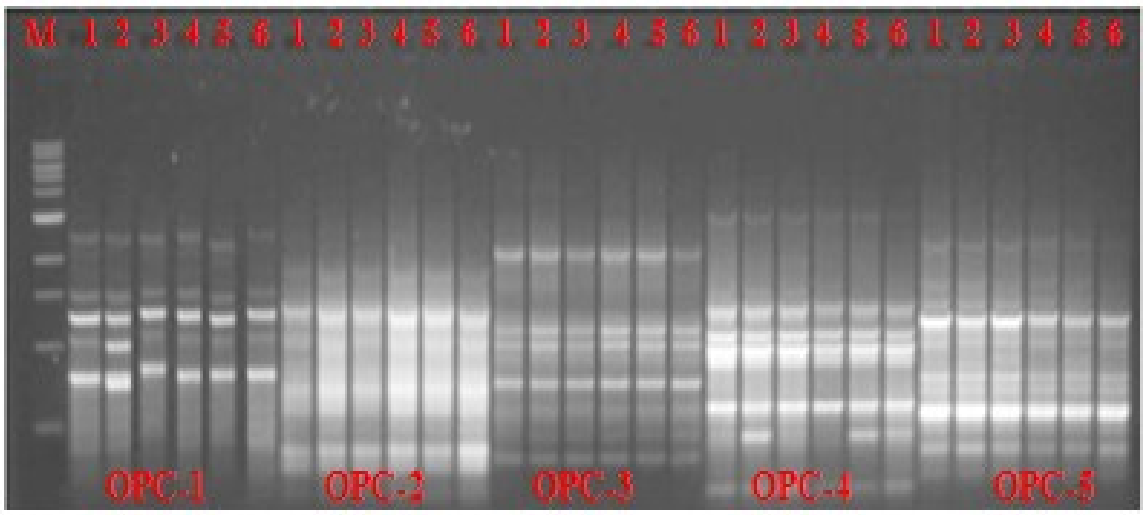


Figure 2.RAPD profile generated by six accessions of Decalepishamiltonii using OPC-1, OPC-2, OPC-3, OPC-4, OPC-5 primers

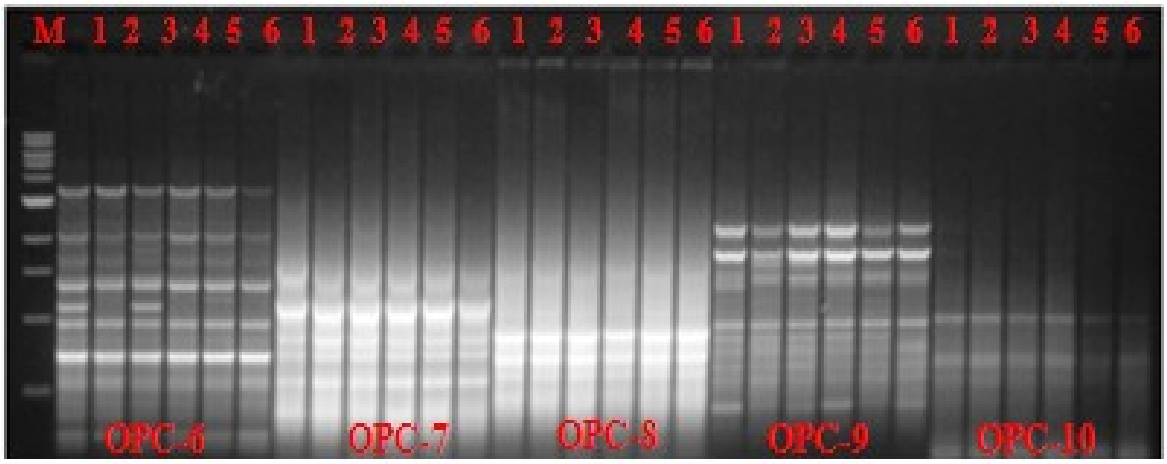


Figure 3. RAPD profile generated by six accessions of Decalepishamiltonii using OPC-6, OPC-7, OPC-8, OPC-9 and OPC-10 primers

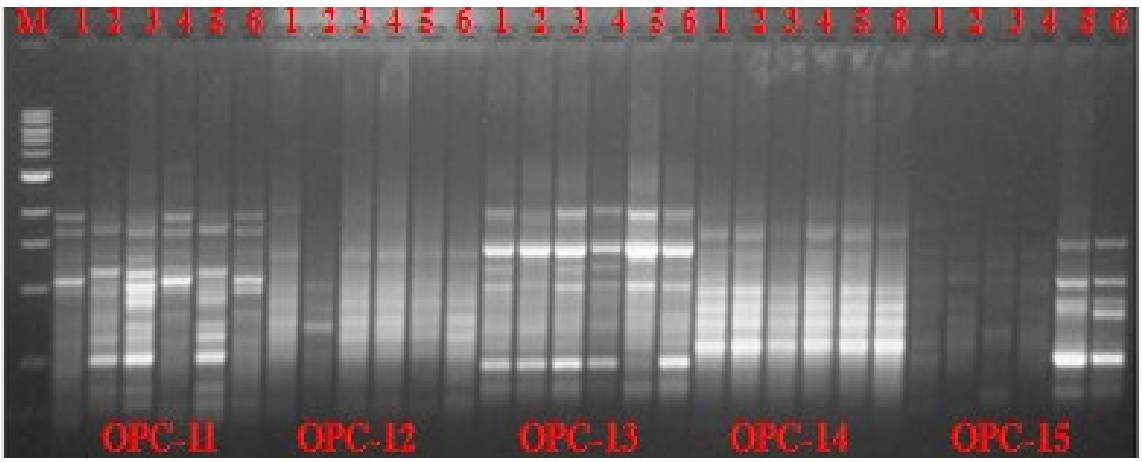


Figure 4. RAPD profile generated by six accessions of Decalepishamiltonii using OPC-11, OPC-12, OPC-13, OPC-14 and OPC-15 primers

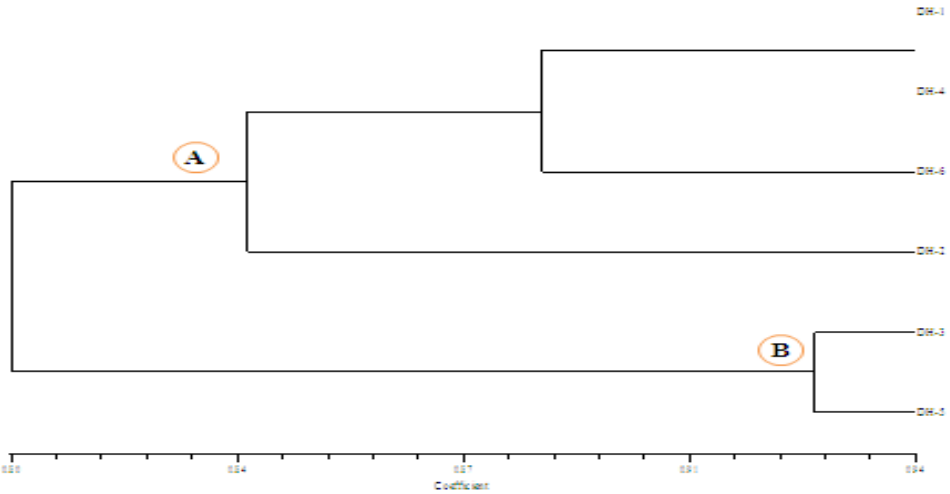
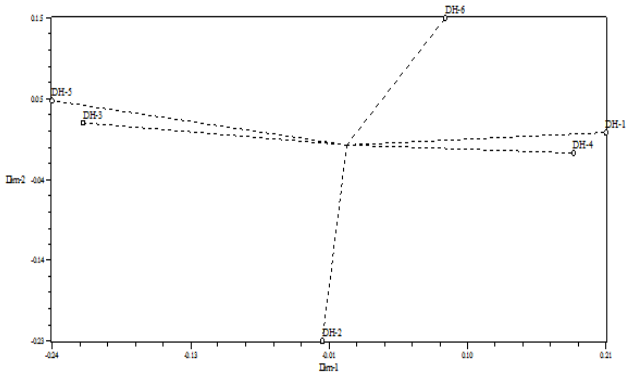


Figure 5. Dendrogram of six accessions of Decalepishamiltonii based on genetic distance generated by fifteen random primers.

Table 3. Distance Matrix calculated by Jaccard`s coefficient of Six Decalepishamiltonii accessions

Accessions code	DH-1	DH-2	DH-3	DH-4	DH-5	DH-6
DH-1	1.0000000					
DH-2	0.8450704	1.0000000				
DH-3	0.7945205	0.8309859	1.0000000			
DH-4	0.9393939	0.8405797	0.7887324	1.0000000		
DH-5	0.7534247	0.8405797	0.9242424	0.7714286	1.0000000	
DH-6	0.8985507	0.8309859	0.8055556	0.8676471	0.8405797	1.0000000

Table 5 Similarity Index (1) Sri Venkateshwara University, Tirupathi, Andhra Pradesh (DH-1), (2) Herbal Garden Agriculture University from, Rajendranagar, Ranga Reddy District, Telangana (DH-2), (3) Pragathi Green Nursery Proddutur Ranga Reddy District (DH-3), (4) Andhra Pradesh Medicinal Plants Board (APMPB), Chilukur, Hyderabad (DH-4), (5) Central Institute of Medicinal and Aromatic Plants from Boduppal, Hyderabad District, Telangana (DH-5) and (6) Botanical garden, Department of Botany, Osmania University, (DH-6).



Figue4.2 D plot diagram- genetic relationships among Decalepis studied.

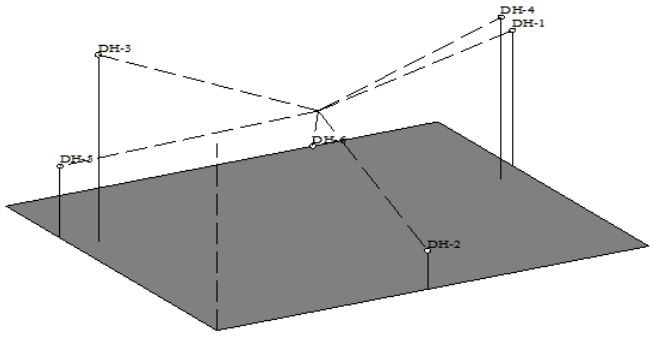


Figure 5. 3 D Plot diagram- genetic relationships among Decalepis studied.

Random amplified polymorphic DNA is one of the important molecular markers for identification of the individual plant species and has provided the polygenetic information and polymorphic index value of the population in a variety of taxonomic and genetic diversity studies. Random amplified polymorphic DNA markers have the greatest advantage of its capability to scan across all regions of genome hence suited for phylogeny studies at species level. This distinction is important to grasp for population studies, particularly when the diversity data is used as a basis for making decisions about conservation of plant resources. For instance, a recent study on some important medicinal plant has showed that Random amplified polymorphic DNA is compatible with morphological data (Mohan et al., 2017). The source of polymorphism observed may be due to deletion, addition or substitution of base within the priming site sequence (Mohan et al., 2017). The present study confirms the suitability of RAPD primers as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of six different accessions types of Decalepishamiltonii collected from different climatic regions. This method can be used for identification of the original Decalepishamiltonii plant. This method can also help distinguish Decalepishamiltonii from other species or adulterants, those results are similar to (Pranay Kumar, 2015; Rama et al., 2015). Despite the small size of the population, which generally exhibit lower levels of genetic diversity, our results based on the Random amplified polymorphic DNA technique showed that the genetic diversity of this critically endangered medicinal important plant species is high.

IV. CONCLUSION

The present study of genetic relationships obtained among the six accessions of Decalepishamiltonii may provide an important source of genetic information for improvement and conservation of this important medicinal plant. Genetic diversity needs to be maintained for the long-term survival of medicinal endangered plant species and it helps the species to adapt to the changing environment. RAPD analysis of the Decalepishamiltonii populations revealed highly variable identified markers, which can be used for population and genetic diversity studies within and between populations. This greatly helps in the identification of superior accessions, or for spotting adulterants and also for taking up major conservation measures of this endangered medicinal plant.

V. ACKNOWLEDGMENT

My thanks to UGC (BSR-RFSMS), New Delhi, for providing fellowship and giving financial support my research work.

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