American Journal of Plant Sciences, 2017, 8, 2119-2132
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

DNA Fingerprinting of Essential Commercialized Medicinal Plants from Pakistan

Waqar Ahmad[1], Khushi Muhammad[1*], Altaf Hussain[1], Habib Ahmad[1], Khalid Kahn[6], Iqbal Ahmed Qarshi[2], Kamran Iqbal Shinwari[1], Muhammad Shahid Nadeem[5], Youxiong Que[4], Ayaz Khan[1], Javed Iqbal[3]

[1]Department of Genetics, Hazara University, Mansehra, Pakistan
[2]Qarshi University, Lahore and Qarshi Industries, Hattar, Pakistan
[3]School of Biological Sciences, University of the Punjab, Lahore, Pakistan
[4]Key Lab of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture, Fujian Agriculture and Forestry University, Fuzhou, China
[5]Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
[6]Women University, Swabi, Pakistan
Email: *khushisbs@yahoo.com

How to cite this paper: Ahmad, W., Muhammad, K., Hussain, A., Ahmad, H., Kahn, K., Qarshi, I.A., Shinwari, K.I., Nadeem, M.S., Que, Y., Khan, A. and Iqbal, J. (2017) DNA Fingerprinting of Essential Commercialized Medicinal Plants from Pakistan. *American Journal of Plant Sciences*, **8**, 2119-2132.
https://doi.org/10.4236/ajps.2017.89142

Received: April 25, 2017
Accepted: August 6, 2017
Published: August 9, 2017

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Abstract

Development of fingerprints based on DNA markers is necessary for proper identification and standardization of plant species. These techniques are widely used to develop an unquestionable method of plant identification to protect the patents and quality control for industry. In this study, fifteen commercially important medicinal plants of Pakistan were collected from botanical garden of Qarshi Industries (Pvt.) Ltd, Pakistan. The objective was to optimize the extraction of genomic DNA for use in a PCR-based random amplified polymorphic DNA marker approach. The initial protocol used 60 decamers to amplify scorable amplicons; only nine markers produced significant bands in genomic DNA of medicinal plants. These markers generated 51 bands ranging between 250 and 1600 bp. The most important property of genomic markers is polymorphism to enable specific identification; all the used markers showed 100% polymorphism across 15 different plants. Further, six decamers amplified specific bands to reliably identify 8 species. The amplified bands were arranged in a binary matrix and analyzed by DNAMAN version 5.2.2 statistical software. A homology tree was constructed using binary data for nine markers, and four major clusters/clades were observed. The Rose, Mentha and Stevia accessions had shown clear clustering and grouped in major clusters/clads I, II and III respectively. Sixty decamers amplified 51 polymorphic loci in the genomes of 15 commercially valuable accessions. Moreover

clear phylogenetic construction was observed in the generation of homolog tree. This protocol could therefore be useful to provide a baseline to authenticate, identify and perform phylogenetic analysis of important medicinal plants used in the Pakistani herbal medicine industry.

Keywords

RAPD, Decamers, Medicinal Plants, DNA Fingerprinting

1. Introduction

Based on the potential therapeutic results of plants, herbal medicines are becoming popular worldwide for human welfare and health [1] [2] [3]. Medicinal plants can be used for different purposes; used as spices/additives to foods, as dying agents, as food, shelter, beverages, insecticides, sweeteners, cosmetics and can be used as bitters and their use is spreading throughout the world including Asia, Latin America and the Pacific countries [4]. It is estimated that about 20,000 species of plants are being used in medicines and the use of extracts derived from the medicinal flora are being used to make allopathic and synthetic drugs [5] [6] [7]. With the development of technology, the therapies for different infections are getting advanced but the use of plants is continued and is one of the main sources to cure ailments [8]. A number of diseases such as pneumonia, ulcers, diarrhoea, bronchitis and catarrh are treated by using different medicinal plants in pure form or in extract forms and new drugs are being developed using medicinal plants to treat AIDS, cancer and various other viral and microbial infections [2] [9]. The Asian countries especially India, Pakistan and Bangladesh have a pool of medicinal plants, as these lands are very fertile having diverse climatic conditions [10]. A huge population uses quite high number of plants and their parts as food, for treating diseases caused by various microorganisms, to apply directly as skin ointments, as decoction and in the form of powder. Hakims and Vadis successfully help the people by preparing the different recipes [11] [12].

The major hurdle in potential use of herbal medicines is the lack of standardization and selection. So, it is necessary to develop the sensitive and effective skills to characterize, identify, authenticate and conserve the medicinal herbs [13] [14]. Proper identification and authentication of plant species is necessary to improve novel medicinal crops [15]. Typically, plants are being identified with the help of Flora of different regions based on visual assessment of morphological and phenological traits in the field [16] [17] [18] [19] [20]. So, it has been difficult to distinguish the plants especially at early stage and at different geographical locations and regional distributions. To protect the patent and quality assurance of plant varieties for industries, it is necessary to develop authentic and unquestionable plant identification methods *i.e.* DNA fingerprinting. DNA-

based molecular fingerprints have acted as very useful tools in various fields like classification; phylogeny, physiology, embryology, plant breeding, population mapping, ecology, genetic engineering etc. and these are based on the polymorphism at molecular level instead of morphological characteristics [20] [21].

Extensive research on molecular markers is in progress in many research institutes all over the world and it is easy, quick and reliable approach to evaluate genetic diversity and determine fingerprints of medicinal plants especially at any stage of development. Recently, many researchers have taken an interest in developing DNA based techniques to authenticate and identify medicinal plants and their raw material [14] [22] [23] [24].

The Random Amplified Polymorphic DNA (RAPD) assay based technology has been widely used by several research groups as an efficient tool for identification of markers linked to agronomically important traits and cultivars, in variability analysis and individual-specific genotyping [25] [26] [27] [28]. For estimation of genetic diversity and DNA finger printing, RAPD technique has been preferably used by researchers and applied to sugarcane [25] [27] [28], potato [29] wheat [26], common bean [30] *Satureja hortensis* L. [31], *Chamomilla recutita* (L.) Rausch.) [32] [33] and *Alicea rosea* [34]. This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer arbitrary. This method appears to be quicker and less labor intensive than the previously used methods such as Restriction Fragment Length Polymorphism (RFLP) analysis.

In present study, a comprehensive effort has been made with an aim to discriminate, authenticate and identify medicinal plants from Pakistan and to increase the efficiency and allow sustainable environment for medicinal industries. It is an elaborative study of fingerprinting of medicinal plants using PCR based DNA markers. Initially, RAPD-PCR technique was adopted to achieve our objective.

2. Material and Methods

2.1. Plant Material

Twenty leaves samples of different commercially important medicinal plants species (each listed in **Table 1**) were collected from botanical garden of Qarshi Industries (Pvt.) Ltd, Hattar, Pakistan. The selected species used in the present investigation were identified with the help of Flora of Pakistan [16] [35] and identification and authentication was confirmed by Altaf Hussain.

2.2. DNA Extraction

The total genomic DNA was extracted from the leaves samples of listed species by using some modification in the standard CTAB protocol of Doyle and Doyle (1990) [36] DNA quantification and quality assessment was done by using Eppendorf spectrophotometry. Normally, quality check was performed through the A260/A280 ratio that is 1.8 values which shows the highest purity [37].

Table 1. The details of medicinal plants accessions used in this study collected from Herb Garden of Qarshi Industries, Hattar, Pakistan.

S. No.	Name	Uses
1	*Rosa wild* Mill.	Antibacterial, antioxidant activity, anti-infective and anti-inflammatory
2	*Rosa bifera* Mill.	Oil is one of the oldest and most valuable perfumery materials.
3	Rose (sahiwal) Mill.	As an important ingredient of potable (sherbet)
4	*Rosa damascena* Mill.	As an antioxidant, laxative
5	*Stevia reboudiana* (Bert.) (Uruguan Source).	The leaf is used traditionally in the treatment of diabetes and substitute to sucrose
6	*Stevia reboudiana* (Bert.) (Canadian Source).	Low calorie substitute
7	*Mentha arvensis L.* Common Mint)	To treat headaches, rhinitis, cough sore throat, colic and vomiting
8	*Mentha piperita (*Peppermint)	Used in tea and for flavoring ice cream, confectionery, chewing gum, and toothpaste etc.
9	*Mentha longifolia* (Horsemint)	Used to treat stomach cramps, asthma, flatulence, indigestion and headaches.
10	*Silybum marianum* (Silybum)	It is used in cases of liver diseases (cirrhosis, jaundice and hepatitis), gallbladder disease.
11	*Viola odorata* (Banafasha)	Blood pressure-lowering effect in rats under anaesthesia.
12	*Nelumbo nucifera (Kanwal)*	To treat ringworm, Skin diseases, cough cold, Asthma.
13	*Ginkgo biloba* L. (Ginkgo)	To treat vascular disease such as intermittent claudication, and in the treatment of cerebral insufficiency
14	*Matricaria chemomile* (Chamomile).	Chamomile has been mentioned in at least in 26 pharmacopoeias of different countries for curing various diseases
15	*Withania somnifera* (Askand-nagori)	Root of this plant is effective in painful swellings, fever and carbuncles.

2.3. RAPD-PCR Primers

For using PCR based markers, different decamer primers were obtained from BioNeer (South Korea) and random decamer primers of the commercial A, C, D and I, J & K-series were acquired for our study Sixty RAPD primers were used initially to investigate molecular basis of the selected medicinal plants. The key novelty of RAPD is the use of a single 10-oligonucleotide arbitrary primer to amplify template DNA without prior knowledge of the target loci. Two basic criteria suggested by Williams *et al.* (1990) [38] must be met for the base pair sequences of RAPD primers *i.e.* minimum of 40% GC content (50% - 80% GC content is generally used) and the absence of palindromic sequence (a base

sequence that reads exactly the same from right to left as from left to right). After primary screening, only those primers giving polymorphic bands were selected for further use (Table 2).

2.4. RAPD-PCR Optimization

For PCR reaction mixture, PCR reaction kit (Enzynomics: Cat# P050B) was used and 20 ul volume of PCR reaction was prepared using IX *Taq* buffer, 2.5 mM $MgCl_2$, 2 mM dNTPs, 10 pmol primer, 0.5UTaq enzyme and 50ng of the isolated genomic DNA. Amplification was carried out in thermocycler (Applied Biosystem 2720, Gradient 96, USA) with initial denaturation cycle at 94˚C for 4 min, followed by 42 cycles consisting of 94˚C for 2 min, annealing at 34˚C for 1 min and extension at 72˚C for 2 min and a final extension cycle at 72˚C for 10 min. Every PCR reaction was repeated thrice to get reproducible results.

2.5. Resolving of PCR Product for Scoring and Data Processing

The PCR amplification products were resolved on 1.5% agarose gel electrophoresis (AGE) with 1× Tris Acetate-EDTA buffer (pH 8.3), stained with ethidium bromide and visualized under UV light (Dolphin Gel Documentation system). The size of the amplicons was estimated from 100 bp to 2500 bp with DNA ladder mix (Thermo scientific, Cat# SM0331).

2.6. Data Analysis

The DNA fragment amplified by RAPD primers were analyzed by size and intensity from all scorable bands. All the data was recorded after scoring RAPD profiles, the number of bands/DNA fragments were represented as present (1) or absent (0) in the genotypes for cluster analysis. The data collected was used to estimate the similarity on the basis of the number of shared amplification products [39]. The similarity coefficients were utilized to generate dendrogram by using UPGMA (Unweighted Pair Group Method of Arithmetic means) through the programme, by DNAMAN statistical software, version 5.2.2 (Applied Biostatistics Inc.).

Table 2. Details of decamers used in present study obtained from BioNeer.

Primer name	Sequence(5'-3')	GC content (%)	Tm (˚C)
A07	GAAACGGGTG	60	32
A09	GGGTAACGCC	70	34`
C08	TGGACCGGTG	70	34
D05	TGAGCGGACA	60	32
D11	AGCGCCATTG	60	32
I08	TTTGCCCGGT	60	32
I16	TCTCCGCCCT	60	32
J08	TCGTTCCGCA	60	32
K01	CATTCGAGCC	60	32

3. Results

In this study, whole genomic DNA was extracted from fresh leaves of the 15 medicinal plants from Qarshi Industries (Pvt.) Ltd. by using modified CTAB method. Some important minor modifications were made in the basic protocol to get best DNA and optimized for best PCR amplification. The highly purified genomic DNA samples from 15 medicinal plants were subjected to analysis and characterization of genomic synteny among them with the help of RAPD-PCR. Sixty RAPD makers were selected from BioNeer kits and applied against the 15 DNA samples. After initial screening, 9 RAPD primers were chosen out of 60 for further study. The selected nine RAPD primers generated 51 scorable amplification products against genomic DNA samples of 15 important medicinal plants (**Table 3**). The results were analyzed by using DNAMAN software on the basis of various parameters *i.e.* total bands (TB), polymorphic bands (PB), monomorphic bands (MP), percentage of polymorphism (PP) and genotype specificity of marker.

The most important application of DNA marker is polymorphism, which can be used to categorize the different plant accessions/genotypes. The selected 9 primers produced 51 detectable amplicons in our DNA samples with the mean of 6.6 loci per primer (**Table 3**). In our study, 100% polymorphism was recorded against the selected medicinal plants and the observed polymorphism produced by decamers could be useful tool to discriminate and identify genotypes. The number of amplified bands/loci ranged from 02 to 09, with the approximate size ranges from 250 to 1600 bp. The maximum number of polymorphic bands (09) produced by primers K01 and F-17 while the minimum numbers of bands were produced by the decamer I16 (**Table 3**).

The specificity of RAPD loci indicated that markers could be used to identify genotypes of important plants. Six RAPD markers had shown specificity with 8

Table 3. The detail of polymorphic and monomorphic bands produced by 9 RAPD Primers in medicinal plants.

Primer name	Sequence	TB	MB	PB	%PP	Band range
A07	GAAACGGGTG	5	0	5	100	350 - 1300
A09	GGGTAACGCC	8	0	8	100	250 - 1500
C08	TGGACCGGTG	6	0	6	100	300 - 1000
D05	TGAGCGGACA	6	0	6	100	300 - 1000
D11	AGCGCCATTG	8	0	8	100	500 - 1600
I08	TTTGCCCGGT	4	0	4	100	300 - 1200
I16	TCTCCGCCCT	2	0	2	100	700 - 1000
J08	TCGTTCCGCA	3	0	3	100	600 - 1200
K01	CATTCGAGCC	9	0	9	100	250 - 1300
Total bands		51				

TB = Total number of bands, MB = Monomorphic bands, PB = Polymorphic bands.

medicinal plants *i.e.* *Withania somnifera* (Askand-nagori), *Gingko biloba* (Ginko), *Nelumbo nucifera* Kanwal, *Withania somnifera* (Askand-nagori), *Mentha longifolia*, *Silybum marianum* (Silybum), *Matricaria chemomile* and *Viola odorata* (Banafasha) by producing 12 band/loci with range of 350 - 1300 bp (**Table 4**).

The homology tree was constructed on the basis of similarity of 15 species of important medicinal plants. A dendrogram was constructed based on Nei's (1978) [40] measures of genetic variance and neighbor joining algorithm of Saitou and Nei (1987) (**Figure 1**) [41]. The statistical software DNAMAN 5.2.2.0 was used to construct homology tree for evaluation of taxonomic values. Our study has revealed that genetic diversity varied among the15 genotypes and ranged from 61% to 96% which is commonly measured by genetic distances or genetic similarity. On the bases of similarity, homology tree discriminated 15 Accessions of medicinal plants into 4 major groups, denoted by roman letters *i.e.* I, II, III and IV (**Figure 1**). In group I, two accessions of genus Stevia shared 96% similarities while three Mentha species were observed to cluster in major group II on the basis of 94% homology. Four different accessions of Rosa were characterized using decamers and these shared between 93% and 94% identity. During phylogeny analysis, these four species/accessions of rose, being commercially used by Pakistani industry, grouped in III cluster. The major group IV clustered four different species of medicinally important plants and wide range of homology ranging from 68% to 76% was recorded among Gingko biloba (Ginko), *Withania somnifera* (Askand-nagori), Kanwal and *Matricaria chemomile* (Chamomile). Moreover,two medicinal plants *i.e.* Banafsha and *Silybum marianum* (Silybum) showed unique fingerprint based on decamers. In the phylogenetic analysis, Banafsha generated 76% identity with group I while Silybum showed 70% similarity with major clusters *i.e.* I, II and III.

Table 4. Specific loci against medicinal plants generated by selected RAPD primers.

Primers	Sequence	Bands (bp)	Specific to
A07	GAAACGGGTG	350	*Withania somnifera* (Askand-nagori)
		400	*Gingko biloba* (Ginko)
		1000	*Nelumbo nucifera* (Kanwal)
		1300	*Withania somnifera* (Askand-nagori)
C08	TGGACCGGTG	400	*Matricaria chemomile* (Chamomile)
		500	*Mentha longifolia*
		900	*Nelumbo nucifera* (Kanwal)
D05	TGAGCGGACA	500	*Gingko biloba* (Ginko)
		1000	*Mentha longifolia*
D11	AGCGCCATTG	1100	*Silybum marianum* (Silybum)
I16	TCTCCGCCCT	700	*Nelumbo nucifera* (Kanwal)
K01	CATTCGAGCC	300	*Viola odorata* (Banafasha)

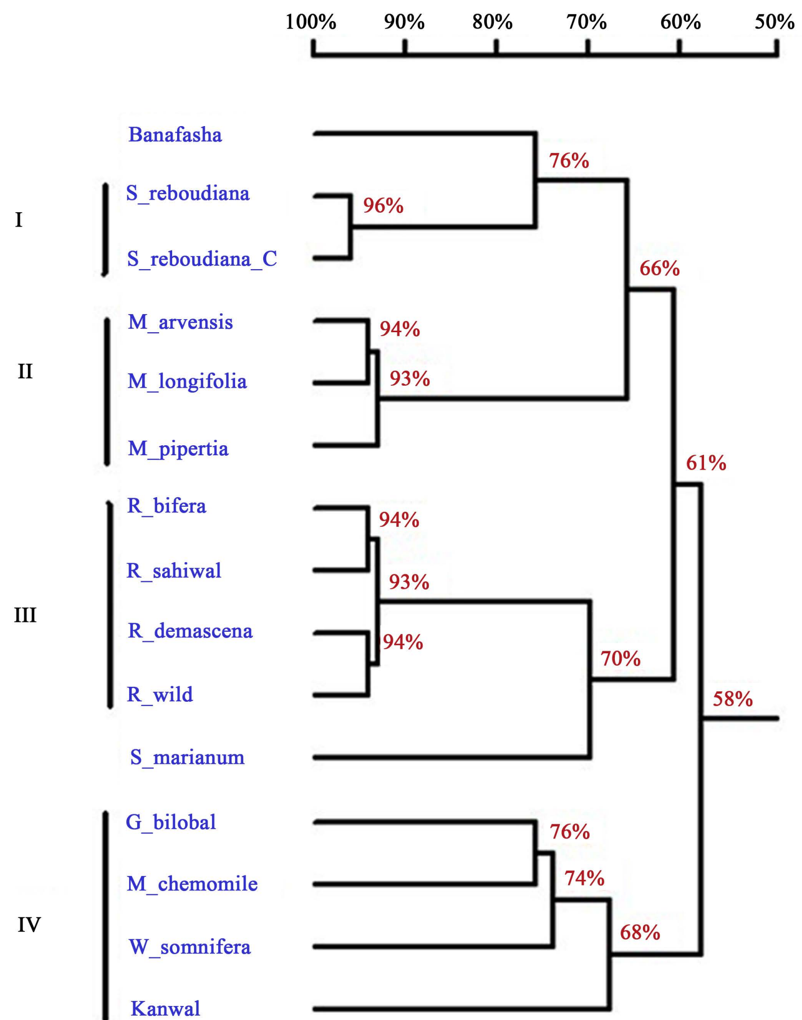

Figure 1. Homology dendrogram constructed showing the genetic similarities among 15 species of important medicinal plants by DNAMAN 5.2.2.0 software based on Nei's (1978) identities/distances (1000 replica). Four major groups were generated by UPGMA. The dendrogram has generated 4 different groups with ranging homology from 68% to 96%. *Stevia reboudiana* (Bert.) (Uruguan Source) and *Stevia reboudiana* (Bert.) (Canadian Source) are grouped in clade I y sharing 96% homology. Three species of mentha namely *Mentha arvensis* L. Common Mint), *Mentha piperita* (Peppermint) and *Mentha longifolia* (Horsemint)are clustered in clade II by sharing homology ranging from 93% to 94%. Rose accessions namely Rosa wild Mill, Rosa bifera Mill. Rose (sahiwal) Mill. and *Rosa damascena* Mill. have shared 94% homology and lustered in group III. Group IV have diverse species/accessions of medicinal plant namely, *Ginkgo biloba* L. (Ginkgo), *Matricaria chemomile* (Chamomile) *Withania somnifera* (Askand-nagori) and *Nelumbo nucifera* (Kanwal). *Silybum marianum* (Silybum) and *Viola odorata* (Banafasha) are showing diversity and are outgrouped.

In this study, we recorded polymorphic properties of RAPD markers in medicinal plants, specific loci to identify plants accessions and phylogeny among different accessions of plants. This is an initial established study to develop and identify unique fingerprints of commercially used plants for medical remedies in Pakistan.

4. Discussion

Medicinal plants play important role in people's livelihood and economics. There are so many species of plants which are medicinal and these are attracted by the markets in Europe and America as herbal medicines. Most importantly, these have contributed to development of Western medicine and ingredients of important drugs. For maximum utilization of the medicinal plants and their extracts, there is prerequisite to identify the plants. Recently, DNA profiling has been used as a versatile technique to investigate genetic variability, genome fingerprinting, gene localization, population genetics, taxonomy and diagnosis.

In the present study, 15 medicinal plants including 4 Rosa, 3 Mentha and 2 Stevia species were obtained from herb garden of the commercial herbal industry (Qarshi Industries Pvt Ltd.) to investigate DNA fingerprints and developing phylogeny using RAPD techniques. Recently, many studies are being conducted to understand origin, identify taxon, developing plant barcodes and understanding conservation of ecology of plants using DNA based marker technologies [42] [43] [44]. We also focused on developing different fingerprints of medicinal plants for industry to make useful and pure products in Pakistan. Prerequisite to run molecular marker against the genome of medicinal plant is to extract high quality DNA from these different plants having many proteins, polysaccharides and phenolic compounds that act as inhibitor against PCR reagents, DNA markers and sensitivity of RAPD markers [45]. We made few modifications in the previously used CTAB methods and overcome the reproducible sensitivity of decamers highlighted by Doyle and Dolye (1990) [36] and Lodhi *et al.* (1994) [46].

During potential marker screening, we selected those generated 100% polymorphism of RAPD primers as a comparable and detectable standard [31]. Wide range of loci size and number were observed to characterize many accessions/species of medicinal plants (**Table 3**). The observed polymorphism produced by decamers could be useful tool to discriminate and identify genotypes [27] [47]. Molecular markers (RAPD) with polymorphic properties have been used to reveal the genetic diversity of many plants having medicinal values especially in the genotypes of chamomile [32] [48]. Previously, RAPD markers approach was used to distinguish diagnose and highlight the genetic diversity in a number of medicinal plants [48] [49] [50] [51].

Proper identification and characterization of plants species from the ecosystem is the most interested objective of this study to develop link between the conservation and utilization of medicinal plant genetic resources. The specificity of RAPD marker was observed to distinguish different accessions of investigating species. Six primers were able to generate species linked loci to identify eight out of fifteen species of medicinal plants (**Table 4**). Potential identification of loci were generated in the *Withania somnifera*, Kanwal, Gingko biloba and *Mentha longifolia* as more than one loci were recorded linked to these species. In past, genotype or variety specific loci generated by DNA markers were reported

to identify varieties of Potato [52], Rhus species [53], fig varieties [54], Jatropha genotypes [55], tea genotypes [56] and *Zingiber officinales* varieties [50].

The genetic similarity was estimated among the fifteen accessions of medicinal plants based RAPD binary data using DNAMAN 5.2.2.0 software. The homology tree was constructed using data generated by nine primers and four major clusters were observed based on similarity. Our results showed constricted variation among different species of Stevia, Mentha and Rose while others species had wide range of variation because of belonging to different generas. Comparatively to our study, using DNA markers, variation at genetic level was investigated among the genotypes or varieties of different plants having medicinal values have been studied by many scientists in wide range of species [48] [49] [57]. The genetic relation and generating DNA based data through RAPD marker was main goal of this study. This technique is widely used because useful properties of RAPD primers and this study were initiated to investigate taxa-specific RAPD bands to identify the species of plants.

5. Conclusion

For identification of fingerprints for medicinally important industrial plant, 60 random decamers were used to amplify scorable loci and highly polymorphic loci were further used to construct phylogenetic tree. Specific loci were identified linked to accessions/species of industrially important plants. Furthermore, 4 clades/clusters were recorded with clear grouping of rose and Mentha species/accessions.

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