Int.J.Curr.Microbiol.App.Sci (2018) Special Issue-6: 2614-2621

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7692 **Special Issue-6 pp. 2614-2621**
Journal homepage: http://www.ijcmas.com

Original Research Article

Development of RAPD Markers for Identification and Authentification of *Embelia ribes*- A Red Listed Indian Medicinal Plant

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A B S T R A C T

Molecular markers play an important role in authentication and identification of medicinal plants. In the present study, RAPD –PCR technique (Random Amplified Polymorphic DNA) has been used to obtain DNA fingerprints of *E. ribes* to distinguish it from other substitutes and adulterants. *Embelia ribes* is one of the important medicinal plants, widely used in preparation of traditional formulations. Different samples of *Embelia* species were collected from various regions of India and RAPD analysis was done using forty seven RAPD primers. Genetic diversity was analyzed among 13 different samples of *Embelia* species collected from different regions of India by using forty seven RAPD primers. Genetic distances were calculated using Jaccard's similarity coefficient. Dendrogram was constructed on the basis of the similarity matrix data by Unweighted Pair Group Method with Average (UPGMA) cluster analysis. In the present study, we have identified three unique polymorphic bands for *Embelia ribes* with three RAPD markers i.e. $OPH4_{530bp}$, $OPH11_{520bp}$ and $OPF5_{480bp}$. These specific bands are absent in the other samples. This molecular characterization is useful for distinguishing *Embelia* from other species and checking the adulteration of *E.ribes*.

Keywords

Embelia ribes, Genetic diversity, Dendrogram, Cluster analysis, Polymorphism, RAPD markers

Introduction

Recently, there is a growing demand for medicinal plants in Industry and also in traditional formulations. However, identification of species from the adulterants is posing a great challenge today. Since, morphological features of medicinal plants are often not intact in the raw drugs. The use of marker chemical compound is also limited, because the bioactive constituents are affected by various other factors. Hence, development of DNA based markers is essential for proper authentication and standardization of herbal preparations. These markers are more reliable, unique, independent of age, physiological and environmental factors. Therefore, the present study of RAPD analysis was undertaken to develop molecular markers for authentication of *Embelia ribes*

Embelia ribes Burm.f (Family-Myrsinaceae) is widely used in several indigenous systems of medicine and in more than 75 Ayurvedic commercial formulations. The plant is a climber with slender branches and long internodes. Leaves are elliptic, broad and covered with minute glands. Flowers are small, white racemes arranged in panicle

inflorescence at the end of the branches. Fruits are berries, round, red to black colour and tipped with style (Anonymous, 1985).The seed resembles so much to pepper and often referred as false pepper and pepper is also used to adulterate *Embelia*.

Embelia ribes, commonly known as Vidanga or Baibirang is one of the important medicinal plants of India. The fruits are astringent, carminative and stimulants. Traditionally, seeds are employed as a remedy for toothache, headache and snakebite. The seeds are mainly used for maintaining healthy skin and to support the digestive function. (Chopra *et al.,* 1966). It is also used as antifungal agent in ringworm infection and other chronic dermatitis. It has laxative activity and used as anthelmintic agent (Singh *et al.,* 1993). Seed extract possess antioestrogenic properties and specially used as contraceptive (Pandey *et al.,* 1993). The seed extract is reported to be antidiabetic (Bandari *et al.,* 2007), antitumour, analgesic, anti-inflammatory (Handa *et al.,* l992; Chitra *et al.,* 2004) antispermatogenic (Seth *et al.,* 1982), chemo preventive (Sreepriya and Bali, 2005) and free radical scavenging activities (Joshi et. al. 2007).

Embelin is the principle chemical compound reported from the seeds of *E.ribes* (*Chauhan et al.,* 1999) Embelin exhibited significant inhibiting activity against some bacteria (Sabitha *et al.,* 2011) and fungal species (Sabitha *et al.,* 2011 and Chitra *et al.,* 2003). *E. ribes* possess close similarities with *E.robusta* in terms of seed morphology and active ingredient *viz.,* embelin, due to this *E. robusta* is used as substitute to *E. ribes* (Pullaiah, 2002).

Previously, *E.ribes* plants are well distributed in Eastern and Western Ghats of India. But, now it is confined to only some packets and one of the red listed species (Ravi Kumar *et al.,* 2000). Because of high demand and low availability, the seeds and herbal formulations of *E. ribes* are contaminated with many cheaper alternatives. Hence, the present study is undertaken to differentiate *E.ribes* from the substitutes and adulterants.

In order to distinguish *E.ribes* from other species, RAPD-PCR markers (Random Amplified Polymorphic DNA) were developed. RAPD is a powerful technique to find the genetic variations existing among several species (Williams *et al.,* 1990). Genetic fingerprinting using RAPD have been widely used for molecular characterization of several plant species (Reyes *et al.,* 1998; Singh *et al.,* 2004). In previous studies, Nair and Kesavachandran. (2006) attempted to characterize 18 accessions of *Gymnema*, using isozyme and RAPD markers. Basha and Sujatha (2007) have reported genetic diversity in a representative set of 42 accessions of *Jatropha curcas* using RAPD markers. Earlier, some RAPD analysis was done to differentiate *E. ribes* from other genus of the family Myrsinaceae i.e *Maesa indica* and *Mysrina Africana (*Devaiah & Venkatasubramanian, 2008). But genetic diversity studies were not reported in *E. Ribes.* In the present study, we have taken up RAPD-PCR technique to distinguish *Embelia* from *E.robusta* and other substitutes and adulterants.

Materials and Methods

Collection of seed sample

A total of 13 different seed samples of *Embelia* were collected from different agro climatic regions of India. Authenticated seed samples of *E.ribes* were collected from M.S

Swaminathan Agro Biodiversity Research Center, Kalpetta (MSSRF), Kerala. Three different seed samples of *E.robusta* were collected from traders of Odisha, Madhya Pradesh, Maharastra and other nine seed samples were collected from local traders which they have collected from different regions of India.

Isolation of genomic DNA and RAPD analysis

Genomic DNA was isolated from *E.ribes* and other seed samples of *Embelia s*pecies by following the protocol of Zheng *et al.*, (1995) with minor modifications**.** The quality and quantity of genomic DNA was checked by running them on 0.8% agarose gel electrophoresis and spectrophotometer.

PCR amplification

PCR amplification reactions (Williams *et al.*, 1990) were performed with forty seven random decamer primers obtained from Operon Technology (Almeda, Calif., USA). For RAPD analysis, the PCR mixture contained 50 ng template DNA, 5 pmoles of each primer, 10 mM dNTPs, 1x PCR buffer (10 mM Tris, pH 8.4, 1.5 mM $MgCl_2$) and 1 unit of Taq DNA polymerase (Genei, Bangalore, India) in a reaction volume of 10µl. Template DNA was initially denatured at 94 ^oC for 3 min followed by 40 cycles of PCR amplification with the following parameters: a 30-sec denaturation at 94^oC, a 30-sec annealing at 36^oC, 60 seconds of primer extension at 72^oC and a final extension at 72^oC for 10 min. The amplified product was electrophoretically resolved on a 1.2% Seakem LE ® agarose gel (Lonza, USA), gels containing 0.5 mg/ml of ethidium bromide in 0.5x TBE buffer and visualized under UV. The 1kb DNA ladder was used as a molecular weight marker.

Data analysis

The amplification profiles of all the 13 samples were compared with each other and a binary matrix was developed, in which 1 represents the presence of a band and 0 represents the absence of band. Pair wise genetic similarity was calculated among the 13 samples belongs to different types of *Embelia* species using Jaccard's similarity coefficient. The similarity matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm on NTSYS-pc (version 2.1, Rohlf1998) software.

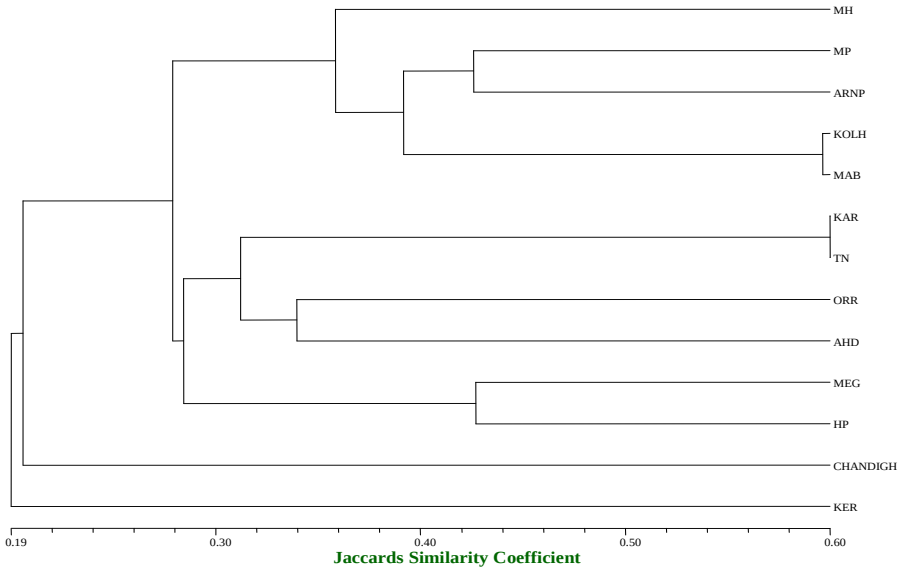
Results and Discussion

In the present study a total of 47 RAPD primers were tested on 13 sample of *Embelia* collected from different geographical regions of India. Out of 47 primers, 22 primers produced clear and highly-reproducible banding patterns. The 22 selected RAPD primers generated a total of 280 alleles with an average of 12 alleles per primer pair. The mean polymorphism information content (PIC) was calculated from the frequency of polymorphic bands across all the *Embelia* species. The primer OPF7 revealed the highest PIC value of 0.96 followed by the primers OPD5 with PIC value of 0.942. The size of the amplified products ranged from 250-3000bp in different samples.

A dendrogram was constructed on the basis of the similarity matrix data by using Unweighted Pair Group Method with Average (UPGMA) Cluster (Fig.1**)** The dendrogram separated the 13 samples into two clusters i.e. Cluster I (CI), cluster II (CII) at 22% level of genetic similarity. Cluster I consist of only one sample i.e. *E.ribes* (Kerala sample), while cluster II

consist of 12 samples of other *Embelia* species. Cluster II was further divided into two sub clusters i.e. sub cluster I and sub cluster II at 26.3% level of genetic similarity. Sub cluster I consist of five samples i.e. Maharastra, Madhyapradesh, Arunachal Pradesh, Kolhapur, Mahabaleshwer and they showed 35 % genetic similarity with SCII. In the sub cluster I, even though the samples were collected from different regions i.e. Kolhapur and Mahabaleshwar they showed high i.e. 58 % of genetic similarity. Sub cluster II was further divided into sub cluster IIa and II b at 32% of similarity. In the sub cluster IIa samples collected from Karnataka and Tamilnadu were showed 60% similarity.

Figure.1 Dendrogram of 13 samples of Embelia using Jaccards similarity coefficient and UPGMA clustering



Seed sample of *Embelia* collected from different regions i.e MH-Maharastra; MP-Madhaya Pradesh; ARNP-Arunachal Pradesh; KOLH- Kolhapur; MAB-Mahabaleshwer; KAR-Karnataka; TN-Tamilnadu. ORR-Odisha; AHD-Ahemadabad; MEG-Megalaya: HP-Himachal Pradesh; CHANDIGH-Chandigarh; KEr-Kerala

Figure.2 RAPD amplification of 13 samples of *Embelia* with primer OPF5

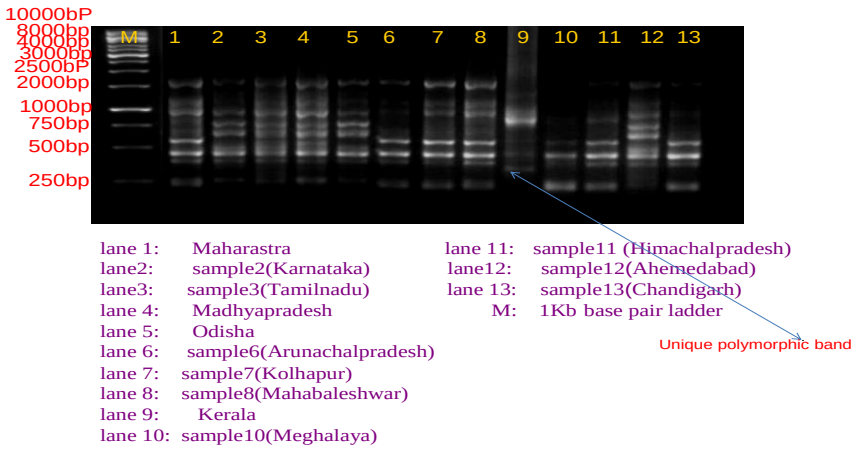
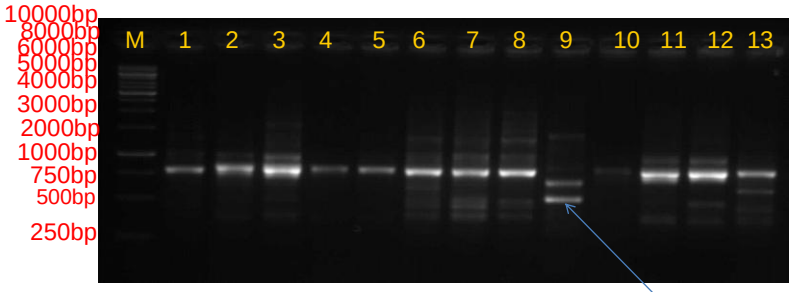


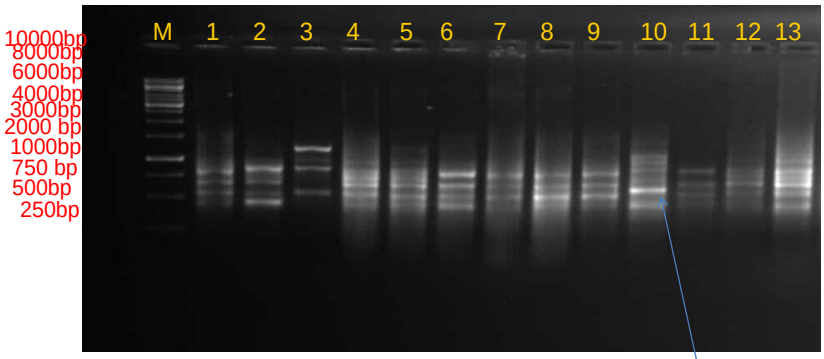
Figure.3 RAPD amplification of 13 samples of *Embelia* with primer OPH11



lane 1: Maharastra
lane2: sample2(Karnataka)
lane3: sample3(Tamilnadu)
lane 4: Madhyapradesh
lane 5: Odisha
lane 6: sample6(Arunachalpradesh)
lane 7: sample7(Kolhapur)
lane 8: sample8(Mahabaleshwar)
lane 9: kerala
lane 10: sample10 (Meghalaya)
lane 11:sample11(Himachalpradesh)
lane12: sample12(Ahemedabad)
lane 13: sample13(Chandigarh)
M: 1Kb base pair ladder

Unique polymorphic band

Figure.4 RAPD amplification of 13 samples of *Embelia* with primer OPH4



lane 1: Maharastra
lane2: sample2(Karnataka)
lane3: sample3(Tamilnadu)
lane 4: Madhyapradesh
lane 5: Odisha
lane 6: sample6(Aurnachalpradesh)
lane7;: sample7(Kolhapur)
lane8: sample8 (Mahabaleshwar)
lane9: sample9(Meghalaya)
lane10: Kerala
lane 11: sample11(Himachalpradesh)
lane12: sample12(Ahemedabad)
lane 13:sample13(Chandigarh)
M: 1Kb base pair ladder

Unique polymorphic band

The analysis of dendrogram has shown that among the 13 samples, four samples of *Embelia* showed high similarity indices. This high similarity index suggests that the samples had close genetic relation among them though they may belong to different geographical regions. Sayed *et al.*, (2009) reported that the genetic similarity of the samples slightly correlated with their close geographic locations. In the present investigation RAPD primers had shown polymorphism between all the *Embelia*

samples. The sources of polymorphism in RAPD assay of different samples may be due to deletion, addition or substitution of base within the priming site sequence (Williams *et al.,* 1990). Similarly, considerable variations at the molecular level were observed in the germplasm of *Gymnema* by Nair and Keshvachandran (2006). The RAPD technique had been successfully used in variety of taxonomic and genetic diversity studies (Rodriguez *et al.,* 1999: Alam *et al.,* 2009). The technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationship in several genera (Wilikie *et al.,* 1993; Demeke *et al.,* 1992; Nair *et al.,* 1999). RAPD markers have the greatest advantage of its capability to scan across all regions of genome hence suited for phylogeny studies at species level (Wilikie *et al.,* 1993; Demeke *et al.,* 1992).

A significant observation in the dendrogram is, *E. ribes* separated as one cluster and did not group with any other species, confirming its genetic distinctness from all other *Embelia* samples. Similar studies were reported in *Jatropha* species i.e *Jatropha grandulifera* remained as a separate cluster compared to other seven *Jatropha* species (Ganesh *et al.,* 2008). In the present study, we have identified three RAPD markers i.e. $OPF5_{480bp}$ (Fig.2), $OPH11_{520bp}$ (Fig.3) and $OPH4_{530bp}$ (Fig.4). These RAPD markers can be used for genetic finger printing and identification of *E.ribes*. The work on further development of the three RAPD primers into a SCAR marker is under progress.

The present study confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of 13 different samples *Embelia* species collected from different regions. The presence of three specific RAPD markers in the samples collected from the Kerala, clearly suggests that these are the original seeds of *E.ribes.* Absence of RAPD markers in other samples suggests there are not original *E. ribes*, may be substitutes with the other species like *E.robusta*. in *E.robusta*. Hence, this methodology can be used for species identification of original *Embelia ribes* and to distinguish it from *E.robusta* and other species of *Embelia* and adulterants. Thus, RAPD analysis has proved as a powerful tool in molecular profiling *Embelia* samples.

Acknowledgments

The authors are grateful to UGC, New Delhi for financial assistance (MJRP No.34-184/2008(SR), Dr. Shivan from M.S.Swaminathan Agro Biodiversity Research Centers, Kalpetta, Kerala for providing the plant material and Dr. Pakki Reddy, Executive Director, Agri biotech Foundation, Hyderabad for providing the lab facilities.

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