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AFLP AUTHENTICATION OF *EMBELIA RIBES* BURM.F AND *EMBELIA TSJERIAM- COTTAM* A. DC.

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ABSTRACT
The two popular and threatened species of *Embelia* viz., *E. ribes* and *E. tsjeriam-cottam* were analyzed for their genetic diversity using AFLP (Amplified fragment Length Polymorphism) studies. The two species exhibited considerable degree of polymorphism up to 88.65%. The EcoRI/MseI enzyme combination did not show scorable bands in stead genome digested and ligated by PstI/ MseI enzyme combination showed scorable bands with unique banding pattern. Species specific markers were also identified during the analysis for both the species which could be further utilized for identification during any circumstances of controversy. AFLP has proven to be robust tool in identifying the degree of similarity and variation between the two important species of medicinal plants.

KEYWORDS: *Embelia ribes* and *Embelia tsjeriam-cottam*, Myrsinaceae, AFLP, DNA fingerprint

INTRODUCTION

Myrsinaceae is one of the important families contributing to the wealth of medicinal plants to a great extent. The well genus of *Embelia* and the species *E. ribes* in particular plays a key role in most of the Ayurvedic and homoeopathic drugs. Among the many species in demand *E. ribes* occupies a prime position both in domestic and international market as it constitutes a bulk of the ingredients used in preparation of ISM&H (Indian System of Medicine and Homoeopathy) and herbal products (Chaudhuri, 2007). *Embelia ribes* locally referred to as Vidanga or Vayu vidanga found distributed through out India upto 1500m in hilly regions of wet deciduous to semi evergreen forests having medicinal properties in roots, leaves and fruit has been considered under threatened category. The roots are acrid, astringent, leaves are thermogenic, demulcent and depurative and fruits are acrid, astringent and bitter (Varier, 1994). Embelin- a benzoquinone derivative is isolated from fruits (Trivedi, 2006). Embelin exhibited significant inhibiting activity against a few bacteria (Gopal and Purushothaman, 1986; Chitra *et al*., 2003). *E ribes* possess close similarities especially in terms of active ingredient viz., embelin with *E. tsjeriam-cottam (syn- E. robusta).* The use of *E. tsjeriam-cottam* is similar to *E. ribes* and it is used as a substitute (Pullaiah, 2002). The fruits are laxative and effective in the treatment of tapeworm and both possess similar characters in fruit and seed morphology (Saldanha, 1984). In order to find the genetic similarities and variations and to identify suitable marker to distinguish the species from one another a molecular tool such as AFLP (Amplified Fragment Length Polymorphism) was employed. It is powerful molecular tool to analyze the genetic difference exiting among several species (Mueller and Wolfenbarger, 1999; Hawkins *et al*., 2005).

MATERIAL AND METHODS

The two species of *Embelia* viz., *E. ribes* and *E. tsejeriam-cottam* were collected from medicinal plant block at Botanical Garden, University of Agricultural Sciences, GKVK campus. The identification of the species was confirmed by referring to the standard floras (Saldanha, 1984). Fresh, young leaves (3 - 4 g) from each species were collected and stored in the deep freezer until further use. Total genomic DNA was isolated by CTAB method (Doyle and Doyle, 1990). Isolated DNA was purified and the concentration of DNA in the samples was determined with agarose gel electrophoresis using λ-DNA as the standard. DNA samples were dissolved in 10:1 TE and stored at -20^{0}C until AFLP analysis. The standard procedure for AFLP was followed with minor modifications (Vos *et al*., 1995). Fifteen enzyme combinations of both EcoRI/ MSeI and PStI/MseI were used for the double digestion of template DNA. The Eco/ Mse adapters and Pst/ Mse adapters were ligated to the ends of restriction fragments. The pre-selective primers have a single base overhang which selects for fragments having extra base downstream of the restriction site.

Pre- Selective amplification

Before selective PCR, a preamplification was carried out to amplify the DNA fragments non- selectively. The PCR programmes was 20 cycles of 30 sec @ 94^{0}C for DNA denaturation, 60 sec @ 56^{0}C for DNA annealing and 60 sec @ 72^{0}C for DNA extension with soak temperature @ 10^{0}C.

Selective amplification

The preamplified product was diluted for selective PCR. Fifteen primer combinations were used (Table 1). The PCR programme was performed for 11 cycles with the following cycle profile. a 30 sec. DNA denaturation @ 94^{0}C, a 30 sec. DNA annealing step and a one min. DNA

extension step @ 72^0C.The annealing temperature in the first cycle was 65^0C which was subsequently reduced in each cycle by 0.7^0C for next 10 cycles. In the next step 24 cycles of 30 sec. @ 94^0C, 60 sec. @ 56^0C and 60 sec. @ 72^0C with soak temperature @ 10^0C. All amplification reactions were performed in MWG thermocycler.

Table 1: Primer pairs used for reamplification *of E. ribes* and *E. tsjeriam- cottam*
(Sl. nos.1-15 corresponds to the numbers given in Fig.1)

Sl. No.	Primer combination
1	E+ACT/ M+CAG
2	E+ACT/M+CAT
3	E+ACG/M+CTT
4	E+TAC/M+CTA
5	E+TAC/M+CAT
6	E+AAC/M+CAA
7	E+ACG/M+CTG
8	E+AGG/M+CTT
9	E+ACC/M+CTG
10	P+GC/M+CTA
11	P+CA/M+CTA
12	P+CG/M+CTA
13	P+GC/M+CTG
14	P+CA/M+CTG
15	P+CG/M+CTG

Gel analysis

Following amplification reaction products were mixed with 20µl of loading buffer (98% formamide. 10mM EDTA pH 8.0, 0.1% BPB and xylene cyanole). Each sample (4 µl) was loaded on a 6% PAGE. The gel matrix was prepared using 40 % Acrylamide, 7M urea and 10X TBE buffer. To 35 ml of gel solution 256 µl 10 % APS and 22 µl of TEMED were added and gels were casted in gel apparatus. 1X TBE was used as running buffer. Electrophoresis was performed at constant power (1500V) for 2 hrs. After electrophoresis gels were fixed in 10 % acetic acid for 30 mins. and washed with milliQ water for 2 min. (3 washes) and kept in silver stain (1g $AgNO_3$; 1.5ml 40% H_2CO) for 30min. The gel was washed in milliQ water for 10sec. only and kept in developer solution (30g $NaCO_3$); 1.5 ml 40% H_2CO; 150µl $NaSO_3$) ultil the bands developed. Then the gel was fixed in 10% acetic acid for 5 min. and washed in milliQ water to remove acid and to prevent gel cracking.

The percentage polymorphism was calculated using the formula

$$\% \text{ polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

RESULTS

The genomic DNA of *E. ribes* and *E. tsjeriam-cottam* digested by EcoRI/ MseI and PstI/ MseI enzymes system and further amplified by 15 different primer combinations as shown in Table 1 gave sufficient variation in terms of number as well as the uniqueness in banding pattern. The nine combinations of EcoRI/ MseI did not show scorable bands. On the contrary, the six combinations of PstI/ MseI gave good scorable bands. A total of 160 bands were scored accounting 142 polymorphic bands out of which seven bands were species specific. The percentage polymorphism contributed to 88.65% (Table 2).

In P+GC/M+CTA combination, three bands ranging between 500-700 base pairs were significant to *E. ribes* and could be considered unique to the species. In P+CA/M+CTG combination two bands between 500-517 base pairs were unique to *E. tsjeriam-cottam*. In P+CA/M+CTG primer combination two significant bands at 200 base pairs was unique to *E. tsjeriam-cottam* (Fig.1). Similar banding pattern did appear with several repeats of the same combination hence could be identified as species specific markers to differentiate the two selected species.

Table 2: Per cent polymorphism between the two species as revealed by the six primer combinations

Sl.No	Primer combination	Total number of bands	No.of polymorphic bands	% Polymorphism
1	P+GC/M+CTA	10	7	70.0
2	P+CA/M+CTA	41	35	85.36
3	P+CG/M+CTA	36	32	88.88
4	P+GC/M+CTG	19	19	100
5	P+CA/M+CTG	25	23	92.0
6	P+CG/M+CTG	31	26	83.87
	TOTAL	162	142	87.65

CONCLUSION

Both the species selected for AFLP viz., *E. ribes* and *E. tsjeriam-cottam* have been found to possess immense potentiality in pharmaceutical industries. Although they exhibit similarity in their active ingredient composition i.e., Embelin performing uniform actions, they do show considerable degree of polymorphism in their genetic makeup as revealed by AFLP analysis. Further the species specific markers generated from the DNA fingerprint can be utilized during any controversy on the identification of species. The identification of molecular markers unique to the plant and stable under different conditions is required for authentication purpose and DNA markers are best suited for this purpose.

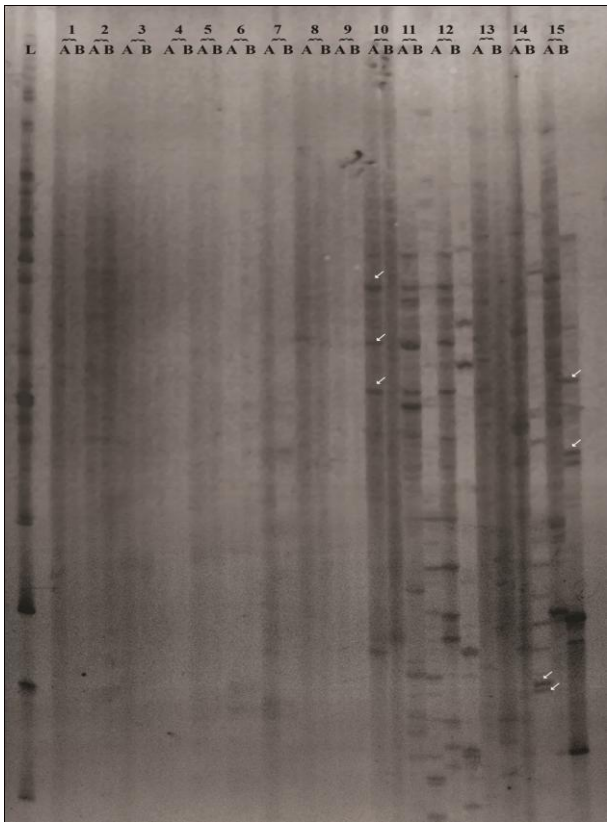

Fig. 1: AFLP fingerprint of *E. ribes* (**A**) and *E. tsjeriam-cottam* (**B**) with 15 primer combinations.

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ABBREVIATIONS

AFLP- Amplified Fragment Length Polymorphism
ISM&H- Indian System of Medicine and Homoeopathy
Syn- synonym
DNA- Deoxyribose nucleic acid
CTAB- Cetyl Trimethyl Amino Bromide
TE- Tris- Ethylene Diamine Tetra Acetic Acid
PCR- Polymerase Chain Reaction
EDTA- Ethylene Diamine Tetra Acetic Acid
TBE- Tris Boric Acid EDTA
$AgNO_3$ – Silver NItrate
H_2CO- Formaldehyde
$NaCO_3$- Sodium Carbonate
BPB- Bromo Phenol Blue