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A STUDY ON THE GENETIC DIVERSITY ANALYSIS OF A MEDICINALLY POTENTIAL HERB- *ENICOSTEMMA LITTORALE* BLUME (GENTIANACEAE)

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ABSTRACT

Enicostemma littorale Blume of Gentianaceae, thriving in many parts of India, South America and some parts of Africa, serves as an important drug for various therapeutics and has been in use traditionally for many diseases. Although, it is identified as an important medicinal plant there seems to be no information on the extent and nature of genetic diversity analysis at the molecular level. Therefore the genetic diversity was analysed using RAPD markers for populations collected from three different regions across Tamil Nadu. Two random decamer primers generated a total of 43 amplified products. The most fragment number amplified by one primer was 26 out of which 7 accounting to 27% were polymorphic in nature. From the RAPD data, an UPGMA dendrogram illustrating the genetic relationship has been computed and the samples were divided into two clusters based on the genetic distance between the samples. The highest and lowest relatedness observed among the samples were based on the distance matrixes which were detected as 0.20 and 0.58, respectively. This relatedness seemed to depend on the nearby location and environmental condition.

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KEY WORDS

Enicostemma littorale Blume, RAPD, Primers, Genetic diversity, Tamilnadu populations.

INTRODUCTION

Medicinal Herbs are making a tremendous revival all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Over three-quarters of the world population relies mainly on plants and plant extracts for health care [1]. *Enicostemma littorale* Blume also called as Vellarugu in Tamil, Mamejavo in Gujarati, Nagajivha in Bengal and Chota chirayata in Hindi is an important group of medicinal plant that belongs to a small herb family-Gentianaceae. It is endemic of Central America and the Caribbean, while the other two species each *E. axillare* and *E. elizabethae* are endemic from tropical Africa and Asia and from Madagascar respectively [2]. The habitat preferences of *Enicostemma* range from very wet to very dry, from savannas to open forests and to radically different levels of humidity. It is an annual or perennial herb.

Enicostemma littorale Blume is an indigenous glabrous perennial rainy season herb, growing on moist, damp and shady ridges and slopes of the borders of cultivated fields, bitter in taste and traditionally used in the treatment of malaria, diabetes, rheumatism, hypoglycemia, blood purifier in dropsy, hernia, swellings, itches, filariasis, abdominal ulcers and insect poisoning and as a stomachic [3]. According to ayurvedic survey, the fresh juice of leaves has been used as a bitter tonic to control arthritis, in typhoid fever and as cooling agent. The plant paste is applied on boils. The leaves are fed to cattle to increase appetite. It is cited in ancient literature as an antimalarial, antipyretic and as a laxative [4]; anti-inflammaotory [5]; anticancer [6]; as stimulant or astringent and diuretic and useful in skin diseases. Both enzymatic and non enzymatic antioxidant are also reported in this plant [7,8]. The plant possesses antihelmintic properties and acts as ehnomedicine for snake bite. It is used to cure leucorrhoea. The root extract showed anti-diabetes activity both invitro and in vivo [9].

Although, *Enicostemma littorale* belongs to an important group of medicinal plants and traditionally been used for many diseases, lacks information about the extent and the nature of genetic diversity information at molecular level that could be obtained using various molecular markers. DNA marker techniques based on PCR amplification have become increasingly important at the study of genetic relationships among plants. Molecular markers provide the best estimates of genetic diversity that are independent of effect of various environmental factors. Various approaches are available for DNA fingerprinting such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), simple sequence repeats (SSRs) and randomly amplified polymorphic DNA (RAPD).

Among these, RAPD is an inexpensive and rapid method not requiring any information regarding the genome of the plant, and has been widely used to ascertain the genetic diversity in several plants by high levels of polymorphism with only small amount of genomic DNA [10]. It provides information that help to define the distinctiveness of species and phylogenetic relationships at molecular level. However, the success of molecular marker and genetic engineering are critically dependent on the development of reliable quality protocol for isolating superior quality DNA and PCR analysis [11] since, the isolation of genomic DNA is a pre-requisite for these molecular techniques. Perhaps, RAPD markers have successfully been used for analyzing genetic

diversity in some plants such as *Vanda*, *Andrographis paniculata* and *Capparis decidua* [12, 13, 14]. These studies have indicated that the RAPD technique is highly potential for identifying the genetic diversity within the population. Hence, realizing the demand, the objective was framed to study the use RAPD markers to identify the genetic diversity present within the isolated population of *Enicostemma littorale* Blume and to use this information for further molecular characterization of this population.

Fig.1

The Geographical distribution of Tamilnadu

The locations from where the plants were collected for the study are represented in the box inside

EXPERIMENTAL PROCEDURES

(i) Plant Material

Fresh leaves of *Enicostemma littorale* were collected from their main naturally growing regions in different provinces across Tamil Nadu (Sathyamangalam, Coimbatore and Erode) (Fig. 1) for DNA isolation. Sampling was carried throughout the year.

(ii) *DNA Isolation*

The fresh plant material was washed in running water, sterilized with double distilled water and powdered with liquid nitrogen and used for extracting total genomic DNA according to the modified procedure of [15], which is actually based on CTAB [16]. About 0.5, 1 and 1.5g each of the freshly prepared powder were added to a warm 65°C (PVPP) / (2-ME/Cetyl-trimethyl ammoniumbromide (CTAB) extraction solution and was incubated at 65 °C for 45 min. To the homogenate mixture was added equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1, v/v) and centrifuged at 12,000 rpm for 10 min. The step was repeated twice. Again to the collected upper phase an equal volume of Chloroform: Isoamylalcohol (24:1, v/v) was added and centrifuged at 12,000 rpm for 10 min. This step was repeated twice. CTAB/NaCl solution [10% (w/v) CTAB; 0.7 M NaCl mixed at 65 °C with stirring] (0.1 volumes at 65 °C) was added to the recovered upper phase and to this an equal volume of Chloroform: Isoamylalcohol (24:1, v/v) was added and centrifuged for 5 min at 12,000 rpm to recover the upper phase. This step was repeated twice. One volume of CTAB precipitation solution [1% (w/v) CTAB; 50 mM Tris–HCl, pH 8.0; 10 mM EDTA] at 65 °C was added to the recovered supernatant. A precipitate formed at this stage was centrifuged at 2700 rpm for 5 min. DNA pellet was recovered by decanting the supernatant and the recovered pellet was dissolved in 500 µL of Highsalt TE buffer [10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA, pH 8.0; 1.0 M NaCl]. The DNA was then precipitated by adding 0.6 volumes of

ice -cold iso-propanol and centrifuged for 15 min at 10, 000 rpm. Subsequently, the recovered DNA pellet was washed in 80% and 100% ethanol respectively. After drying, the pellet was re-suspended in TE buffer [10 mM Tris–HCl, pH 8.0; 1.0 mM EDTA, pH 8.0] and stored at -20°C until further use. Total DNA was quantified spectrophotometrically (Shimadzu 3600 Spectrophotometer) and a sample yielding good quality was determined by calculating the ratio of absorbance at A_{260}/A_{280} ratio [17, 18].

(iii) ***RAPD-PCR amplification reaction***

RAPD-PCR was carried out using modified procedure of [19] but by using two random decamer primers that were purchased from a commercial source (GeNei, Bangalore).The reaction mixture of 50µL consisting of 2.0µL of DNA, 8.0µLof primer (2µM/µL), 5.0µL of dNTP (2mM), 0.5µL of *Taq* DNA polymerase (5U/µL), 5.0 µL of 10X Buffer and 29.5µL Sterile distilled water were amplified in a thermo cycler (EPPENDORF Mastercycler) that was programmed with 5 min at 94º C for initial denaturation, followed by each 34 cycles of 40 sec at 94º C and 30 sec at 55º C respectively, with an extension time at 72º C for of 1min and 10 mins separately followed by a hold temperature of 4ºC at the end. After amplification, the DNA fragments were separated by electrophoresis for about 3 hour under constant voltage (75V) in a 2% agarose gel submerged in 1X TBE buffer (89mM Tris base, 89mM Boric acid, 2 mM EDTA) and visualized with ethidium bromide and photographed using Gel Documentation unit (VILBER LOURMAT, FRANCE). A 100 bp ladder (Bangalore gene, India) was used as the molecular marker.

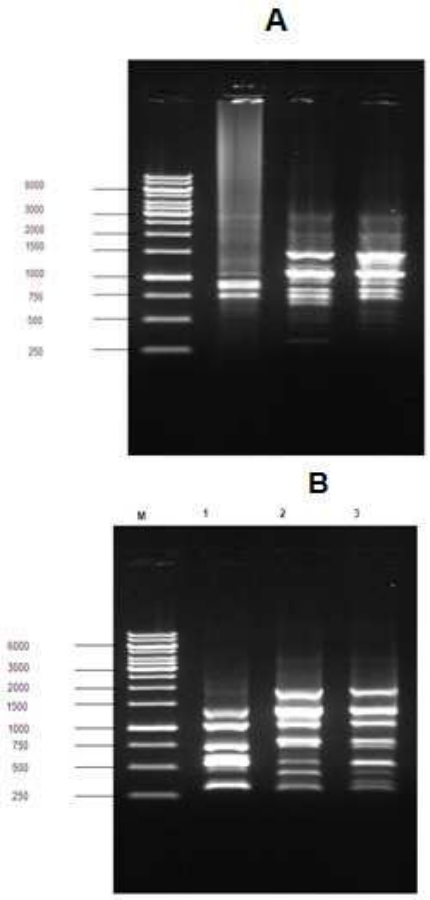
(iv) ***Statistical Analysis***

The amplified fragments were characterized by size and intensity for all scorable bands. The presence or absence of fragments was indicated as (1) and (0) respectively [20] according to whom, the binary matrix was calculated based on the scoring data. The binary matrix of the three samples obtained was converted into the distance matrix using R Language software. The distance matrix obtained was used to generate a dendogram using UPGMA method (Unweigthed Pair Group Method Arithmetical Means) and viewed using NJ Plot to calculate the distance between the branches.

RESULT

Various optimization of RAPD-PCR reaction parameters were carried out with two random decamer primers (OPC11 and PG07) that were found suitable for the RAPD applications. The random primers OPC11 and PG07 possessed 60% GC content each with nucleotide sequences 5'AAAGCTGCGG 3' and 5'GCTGCAGTAG 3'respectively. Though two primers generated a total of 43 amplified products, the primer OPC11 showed high level of polymorphism compared to PG07. Primer OPC11 individually generated a total of 26 amplified products of which 8 fragments were amplified from sample of Sathyamangalam, while 9 each for samples of Coimbatore and Erode respectively. This primer amplified an average of 5 high intensity fragments for each sample which showed the presence of high level of DNA. Out of 26 amplified fragments, 7 fragments accounting to 27% were found polymorphic while the remaining 19 fragments accounting to 73% were identified as monomorphic. In contrast to the above, the primer PG07 generated only a total of 17 amplified products and showed an average of 2 high intensity fragments for each sample. However, the size of amplified products ranged from 2000 to 250 base pair for the primer OPC11 and from 3000 to 500 base pair for primer PG07 respectively (Fig 1A & B).

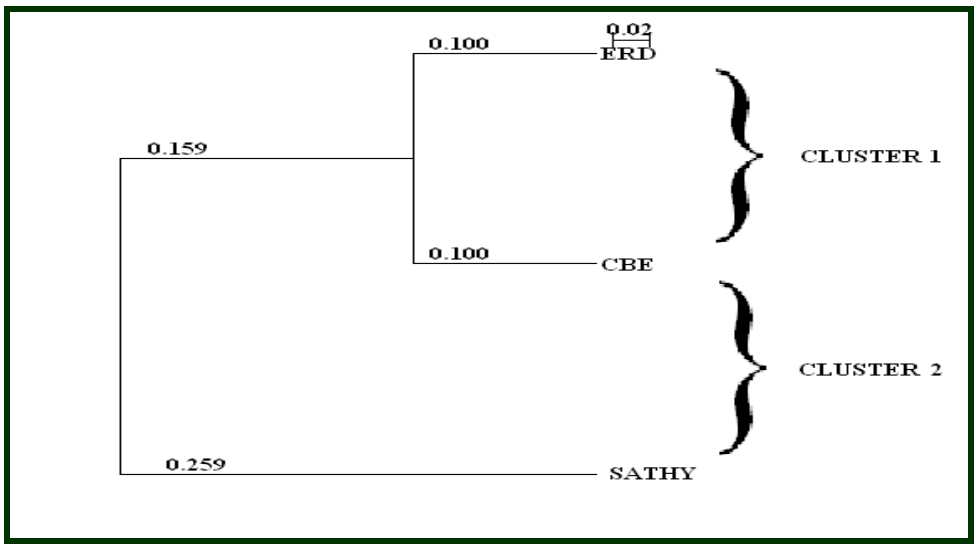
Figure 1
Amplification profile of E.littorale accessions with RAPD decamer pimers



Shows the comaparative amplication profile between the samples used with the primers.
A. RAPD-PCR profile of E.littorale sp. collected from three different using OPC11 decamer primer
B. RAPD-PCR profile of E.littorale sp. collected from three different using PG07 decamer primer
M: 10 kb DNA ladder, Lane 1: Sathyamangalam sample, Lane 2: Coimbatore sample, Lane 3: Erode sample

From the RAPD PCR result, the amplified bands were scored (1) and (0) based on the presence or absence of bands and was indicated as the binary matrix for calculation, which were then converted into distance matrix using R-language software, where the distance matrix was calculated and from the distance matrix, a dendrogram was generated using the UPGMA method (Unweigthed Pair Group Method Arithmetical Means) that was visualized using NJ plot tree viewer (Fig 2).

Figure 2
Dendrogram obtained from RAPD analysis using UPGMA.



Cluster analysis of E.littorale samples based on RAPD data using UPGMA. The Value on branches represents the genetic distance among samples.
ERD -Erode sample, CBE - Coimbatore sample, SATHY- Sathyamangalam sample.

The dendrogram obtained using UPGMA as rooted phylogenetic tree revealed that the samples collected from Coimbatore and Erode were highly related which yielded a genetic distance of 0.2 whereas sample collected from Sathyamangalam showed a genetic distance of 0.418 indicating a slight variance from Coimbatore and Erode samples.

DISCUSSION

Genetic diversity of different materials can be studied by using molecular marker techniques like RFLPs, RAPDs or AFLPs. The technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationship in several plants [21-23].The major concern for RAPD-generated phylogenies include homology of bands, which show the same rate of migration, causes of variation in fragment mobility and origin of sequence in the genome [24]. Thus, RAPD markers have the greatest advantage to scan across all regions of the genome proving its capability as highly suitable for phylogeny studies at species level [21, 22].

The present investigation revealed genetic relatedness among the accession of *E.littorale* and this has led to the establishment of highest and the lowest genetic relatedness among the accession collected from various regions. The highest genetic relatedness was found between the accession CBE (Coimbatore) and ERD (Erode) which possessed lowest distance of 0.2 and followed by SATHY (Sathyamangalam) and ERD (Erode) that possessed the distance of 0.45. The lowest genetic relatedness was observed between SATHY (Sathyamangalam) and CBE (Coimbatore) because it possessed highest distance of 0.58. The highest and the lowest relatedness observed among the samples were based on the distance matrixes which were identified as 0.20 and 0.58, respectively.

The UPGMA dendrogram computed the data obtained by grouping the samples in two clusters. Thus the sample collected from Coimbatore and Erode were genetically more related as judged by the observation that they group together in one cluster in the dendogram, while the sample collected from Sathyamangalam was genetically less related and was found in other cluster. This also indicated that sample from Sathyamangalam could have evolved first and from this Coimbatore and Erode sample could have been evolved. Furthermore, it is suggested that the highest relatedness between the samples collected from Coimbatore and Erode seemed to depend on the nearby location and perhaps, the same environmental conditions. Moreover, the lowest relatedness however could be reflected by both the global variation of the species and reproductive biology of the species. Perhaps, the use of primers, where primer OPC11 produced good amplification product when compared to PG07, which did not exhibit any discrete pattern for evaluation even after repetitive trials indicated that there was no polymorphism with the use of primer PG07. Hence it can be proposed that the location with nearly similar climatic conditions might impose limited variation in genetic diversity of the plant in general and that could be attributed to *Enicostemma littorale* too. Anyway, we presume that to the best of our knowledge, this would be the first report of the kind on evaluation of genetic diversity analysis for this plant (*Enicostemma littorale* Blume) especially by applying RADP analysis as a valuable input providing way for the start of further studies by more powerful markers.

CONCLUSION

Experimental results evidenced the role of RAPD markers, which allowed estimating the genetic diversity for *Enicostemma littorale* Blume. In this present work RAPD analysis has provided the information on the genetic relatedness and phylogenetic relation within the isolated population *Enicostemma littorale* Blume.

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