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MORPHOLOGICAL AND MOLECULAR POLYMORPHISM AMONG THE MEDICINAL HERB *CASSIA OCCIDENTALIS* L. ACCESSIONS FROM THIRUVANANTHAPURAM, KERALA

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ABSTRACT

Cassia occidentalis L. is a pantropical, traditional herbal weed that invades as impenetrable thickets in open land with variable morphoforms. The taxonomic complexity of this species is caused in part by the phenotypic plasticity of the species and leads to natural intra-specific hybridization. An initial attempt was made to differentiate the accessions collected from ten localities of Thiruvananthapuram, Kerala in terms of morphology and molecular techniques. Leaf length, width and plant height showed variability. The genetic diversity of ten accessions of *C. occidentalis* collected from different localities were analyzed using twelve RAPD primers. 111 bands were scored corresponding to an average of 9.25 bands/ primer with 79 bands showing polymorphism (71.17%). 9 out of 12 primers gave more than 60% polymorphism. Jaccard similarity coefficient ranged from 0.54 to 0.73. A dendrogram constructed based on the UPGMA clustering method revealed two major clades. Clade 1 comprises of 8 accessions which was further differentiated into two sub-clusters while Clade 2 includes two accessions. This study showed significant genetic polymorphism among *C. occidentalis* accessions.

KEYWORDS: *Cassia occidentalis,* Molecular analysis, SDS-PAGE, Dendrogram, Morphological.

INTRODUCTION

Cassia occidentalis L. a shrub belonging to Fabaceae. It grows throughout the tropics and subtropics. It reaches heights of about two meters with yellow flowers in the leaf axils. It is used for landscape purposes, as food and medicinal. Previous pharmacological investigations showed that *C. occidentalis* leaf extracts have broad spectrum antibacterial, antimalarial, antifungal, antimutagenic, antiplasmodial, anticarcinogenic, and hepatoprotective activity. It is also used against stomach disorders, rheumatism and in treatment of liver diseases. The leaves and roots are ingredients of many popular herbal liver tonics, medicines for liver and stomach disorders. Moreover, the leaves are widely used as a leafy vegetable and eaten either raw or mixed with coconut, chilly and onion. In addition, it has been used for the control of a large variety of insects and used also to reduce the number of mosquitoes indoors at night. Genetic variation in a population is considered to represent its evolutionary potential. Genetic variation has implications for conservation at the species level. These molecular markers can characterize plants with greater precision than biochemical parameters.[1] Species genetic diversity can be interpreted under two criteria. One is the allelic richness, which is measured as the total number of different alleles at each locus in a population or species. A second factor that also accounts for diversity is the evenness of allele frequencies in each locus, and this is measured along with allelic richness by the coefficient of gene diversity. There are various types of DNA markers like Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeats (VNTRs), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD). Among these, RAPD markers are efficient to assess genetic variation and have been used extensively to evaluate natural genetic diversity in plant populations, phylogeny and systematic, genetic linkage mapping and gene tagging. The presence or absence of one RAPD band is diagnostic of variation in the sequence within the primer binding sites in the target genome. This marker system has the ability to amplify DNA from dispersed polymorphic loci and has its power to detect small genetic differences.

Therefore, the pinpoint objective of the present investigation was to carry out a morphological and genetic polymorphism study of *C. occidentalis* accessions under local conditions to get a better insight of variations in this medicinal herb.

MATERIALS AND METHODS

Plant Materials

Fresh and healthy *Cassia occidentalis* L. plants were collected from 10 different localities of Thiruvananthapuram District viz., Parassala, Amaravilla, Neyyattinkara, Kattakada, Aruvikkara, Palode, Vembayam, Kazhakuttam, Aryanadu and Attingal.

Methodologies

Morphological Analysis

Distinct populations were selected from the 10 regions. From each location, 5 to 20 plants were randomly selected, based on differences in morphological characteristics. Morphology of fertile aerial parts of plants was analyzed under stereoscopic microscopic Olympus SZH, coupled to an Olympus C-35AS-4 camera. Morphological characteristics like height of plant, leaf length, breadth, leaf area (graph paper method), and thickness of fruit rind were measured. The data were analyzed using Analysis of Variance (ANOVA). Branching pattern (parallel or drooping), leaf apex (blunt or acute), leaf colour and fruit colour was also recorded for all four populations.

Molecular

Genomic DNA was extracted from fresh leaves of *C. occidentalis* and quantified as per the protocol of Doyle and Doyle [2] with minor modifications. Williams et al. [3] method was adapted for PCR reaction with 10-mer oligonucleotides synthesized by Operon technologies. The final volume of 25µl contained 10 x buffer with $MgCl_2$, 20 ng of genomic DNA, 0.25 mM dNTPs, 100 µM of primers, 1.5 mM $MgCl_2$ and 1U of *Taq* polymerase. Amplification was carried out in thermocycler, cycle 1 comprise 1 min at 94°C followed by 44 cycles, each consisting a denaturation step for 1min at 94°C, followed by an annealing reaction for 1min at 36°C and an extension step for 2 min at 72°C, followed by a further extension step for 5 min at 72°C and the samples were cooled at 4°C. Samples of 10 µl RAPD-PCR product were subjected to 1.4% agarose electrophoresis and the amplified products were stained by ethidium bromide for detection.

A dendrogram was constructed by using the UPGMA with SAHN module of NTSYS software to show a phenetic representation of genetic relationship as revealed by the similarity coefficient.[4]

Statistical Analysis

The data was statistically evaluated by one way ANOVA and t-test. The results are average of 6 replications and are represented as mean ± SD.

RESULTS AND DISCUSSION

Due to cosmopolitan distribution, *C.occidentalis* show varied adaptations to acclimatize with the different natural environments. Here an attempt was made to examine the *Cassia occidentalis* accessions collected from ten different localities of Thiruvananthapuram, Kerala for species delimitation with morphological and molecular markers.

Morphological Analysis

Morphological characters such as leaf length, breadth, leaf apex, leaf area, stem height, stem girth, fruit colour, and rind thickness showed profound variability (Tables 1 and 2). The leaves from the Attingal region had broad lamina (5.73 cm) with leaf length of 12.8 cm (Table 2) while that of Kazhakuttam population were long (16.3 cm) with 4.9 cm breadth (Table 2). Likewise, the populations from Parassala, Amaravilla, Neyyattinkara, Kattakada, Aruvikkara, Palode, Vembayam, Aryanadu were different from that of Attingal. These plants were comparatively smaller. Leaves had broad lamina and acute apex. The population from Attingal was also significantly taller with longer dark green leaf lamina and acute apex. Although cosmopolitan species show high levels of genetic variability due to narrow genetic drift and strong selection of dominant characters in controlled environment, insect pollinated, seed raised progenies exhibited lots of variability in height, branching pattern and leaf and fruit morphology. It is necessary to examine whether the morphological diversity in plants at different region existed at molecular level or it is mainly due to environmental conditions.

Table 1. Morphological diversity (mean ± SD) in different populations of *C. occidentalis* accessions. Significant at $P < 0.005$.

Accession	Leaf Length (cm)	Leaf Width (cm)	Petiole Length (cm)	Inter node length (cm)	No. of fertile stamens	Filament length (cm)	Carpel length (cm)	Style Length (cm)	No. of ovules	No. of fruits/ bunch
Palode	4.8±0.3	3.1±0.3	0.1 ±0.0002	1.7 ±0.2	7 ±0.2	0.35 ±0.1	1.3 ±0.3	0.3 ±0.1	32±0.3	3 ±1
Parassala	4.3±0.3	2.9±0.3	0.2 ±0.001	1.5 ±0.3	7 ±0.3	0.35 ±0.1	1.1 ±0.3	0.2 ±0.2	42±0.2	3±1
Neyyattinkara	3.9±0.2	1.8±0.1	0.2 ±0.002	2.2 ±0.1	7 ±0.3	0.3 ±0.2	1 ±0.4	0.5 7 ±0.2	40±0.5	3 ±1
Aruvikara	4.5±0.2	2.5±0.1	0.2 ±0.001	1.5 ±0.1	7 ±0.2	0.3 ±0.1	1.2 ±0.5	0.4 7 ±0.2	30±0.1	2±0.5
Vembayam	4.8±0.3	3.2±0.2	0.1 ±0.001	2 ±0.1	7 ±0.2	2.5 ±0.1	1.5 ±0.2	0.4 ±0.2	37±0.3	3±1
Kazhakuttam	5.2±0.3	2.3±0.2	0.2 ±0.001	2.5 ±0.1	7 ±0.2	0.38±0.1	1.5 ±0.3	0.5 7 ±0.4	27±0.3	3±1
Amaravilla	3.8±0.01	1.67±0.1	0.18±0.001	2.3 ±0.4	7 ±0.2	0.32 ±0.1	0.9 ±0.08	0.527 ±0.2	38±0.08	3 ±1
Kattakada	3.9±0.09	1.88±0.1	0.17 ±0.001	2.2 ±0.5	7 ±0.2	0.32 ±0.2	0.89 ±0.04	0.497 ±0.2	37±0.22	3 ±1
Aryanadu	3.8±0.04	1.98±0.1	0.19 ±0.001	2.42 ±0.3	7 ±0.2	0.32 ±0.1	0.1 ±0.03	0.537 ±0.2	39±0.34	3 ±1
Attingal	5.5±0.3	2.5±0.2	0.24 ±0.001	2.7 ±0.7	7 ±0.2	0.37 ±0.1	1.7 ±0.2	0.557 ±0.2	29±0.84	2±0.5

Table 2: Variations in morphological characteristics of *C. occidentalis* accessions. Significant at *P* < 0.005.

Accessions	Stem girth (cm)	Branching point (cm)	Type of branching	Leaf Apex	Leaf Colour	Fruit Colour
Palode	5.8±0.98	1.1±0.3	Parallel	Blunt	Green	Green
Parassala	6±0.53	1.4±0.3	Parallel	Blunt	Green	Green
Neyyattinkara	5.9±0.12	0.8±0.1	Parallel	Blunt	Green	Green
Aruvikara	5.5±0.52	0.5±0.1	Parallel	Blunt	Green	Green
Vembayam	6.98±0.63	1.2±0.2	Parallel	Blunt	Green	Green
Kazhakuttam	11±0.21	2.3±0.2	Drooping	Acute	Greenish yellow	Green
Amaravilla	6±0.01	0.87±0.1	Parallel	Blunt	Green	Green
Kattakada	7±0.09	0.88±0.1	Parallel	Blunt	Green	Green
Aryanadu	7.78±0.04	1.5±0.1	Parallel	Blunt	Green	Green
Attingal	13.5±0.67	3.5±0.2	Drooping	Acute	Greenish yellow	Green

Molecular Analysis

Out of fifty primers used for screening, 38 did not amplify any fragment (Fig. 1 a, b, c, d), whereas, other 12 primers generated amplicons ranging from 3 (OP 177) to 13 (OP 106 & OP 112). The reproducibility of the bands generated by these 12 primers was confirmed by replicating the amplification trice. Only the bands showing reproducible amplicons were considered for scoring and for further analysis. The number of polymorphic bands ranged from 3 to 13 with range of polymorphisms 37.5% (OP 61) to 100% (OP 178, OP 121, OP 171). 111 bands were generated by twelve amplifying primers with an average amplification of 9.25 bands/ primer. Mean polymorphism produced by these bands was 71.17%. Amplicons size generated varied from 385 to 3400 bp. In the present study, the PIC ranged from 0.166 (OP177) to 0.394 (OP 121).

Jaccard's pair wise similarity coefficient values ranged from 0.54 to 0.73 (Table 3). The clusters constructed through NTSYS (2.02 PC) presented in the form of dendrogram has been shown in Figure 2. Dendrogram grouped all the genotypes in to two major clades (group I and II). Clade I comprises of eight accessions which was further grouped into two sub-clusters (S1& 8, S6 & 7, 9; S3 & 4, S10) while clade II includes two accessions (S2 and 10). The genetic diversity of the plants is closely related to their geographic distribution. Species with a wide geographic area generally have more genetic diversity. The present RAPD analysis showed high genetic diversity in *C. occidentalis* accessions growing in different environments and low diversity in *C. occidentalis* accessions in the same or adjacent regions, with a few exceptions. For example, *C. occidentalis* accession C 4 and C 6 are from

Kattakada and Palode respectively, which are not geographically much far apart, but their genetic distance was great in the present RAPD analysis.

In the present study, the medicinal plant *C. occidentalis* showed a high percentage of genetic polymorphism of 71.17%, which was near to the percentage for *Achillea fragrantissima* [5] (69%) ,*Artemisia capillaries* [6] 73.33% but higher than that of *Garcinia indica* [7] (19.93%) and *Commiphora wightii* [1] (28%). Similarly, the genetic diversity index was also highly variable from 0.54 to 0.73 in case of *C. occidentalis* accessions. These studies indicate that RAPD is sufficiently informative and powerful to access genetic variability of natural populations of *C. occidentalis.* Thus, RAPD markers will provide a useful tool in the future design of collection strategies for germplasm conservation.[8,9]

During intraspecific genetic variability study, the divisions of 10 samples of *C. occidentalis* into two clades as shown in Figure 2 allowed very little chances for gene flow among accessions that were geographically distant, but the probability of naturally occurring genetic cross and gene flow should be high among accessions growing near each other. So, this study concluded that the high genetic diversity among accessions in adjacent regions was mostly attributable to artificial introduction, not natural genetic differentiation.[10,11]

RAPD, being a multi-locus marker with the simplest and fastest technology, have been successfully employed for determination of intra-species genetic diversity in several plant species.[12,13] In case of *C. occidentalis,* C 10 sample from Attingal did not group with any other accession in dendrogram confirming its genetic distinctness from all other accessions included in this investigation.

The calculated PIC based on the probability that two unrelated genotypes amplified from the test population will be placed into different typing groups. PIC estimates the degree of polymorphism of marker, which essentially is the proportion of individuals that are heterozygous for a marker. PIC is a good measure of the heterozygosity. It is an index of how many alleles a certain marker has and how those alleles divide. High PIC value indicates rich heterozygosity which in turn is associated with a high degree of polymorphism.[14,15] In case of *C. occidentalis,* good range of PIC value was observed, which showed significant genetic diversity among *C. occidentalis* accessions.

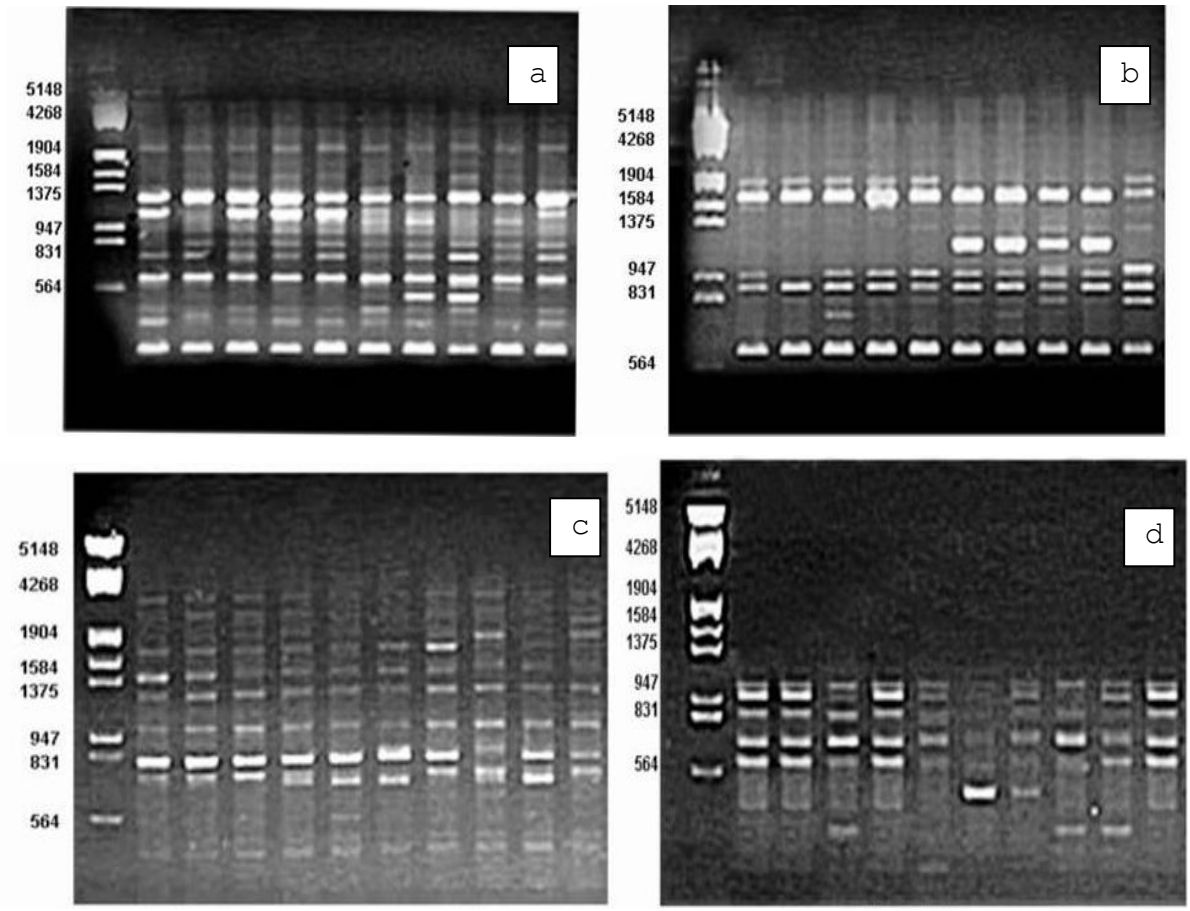

Figure 1a, b, c, d: Genetic polymorphism among the accessions of *C. occidentalis* using different RAPD primers.

Table 3: RAPD primers showing total and polymorphic bands generated along with PIC of each pattern for 10 accessions of *C. occidentalis.*

Primers	Total no. of bands	Total no. of polymorphic bands	% Polymorphism	PIC
OP 106	13	8	61.5	0.242
OP 23	10	7	70	0.183
OP 103	6	5	66.7	0.211
OP 112	13	4	38.5	0.173
OP 61	8	3	37.5	0.227
OP 104	8	6	75	0.246
OP 178	9	9	100	0.241
OP 135	11	8	73	0.261
OP 69	11	9	82	0.394
OP 121	11	11	100	0.166
OP 177	3	2	66.7	0.342
OP 171	7	7	100	0.212
Total	111	79	71.2	

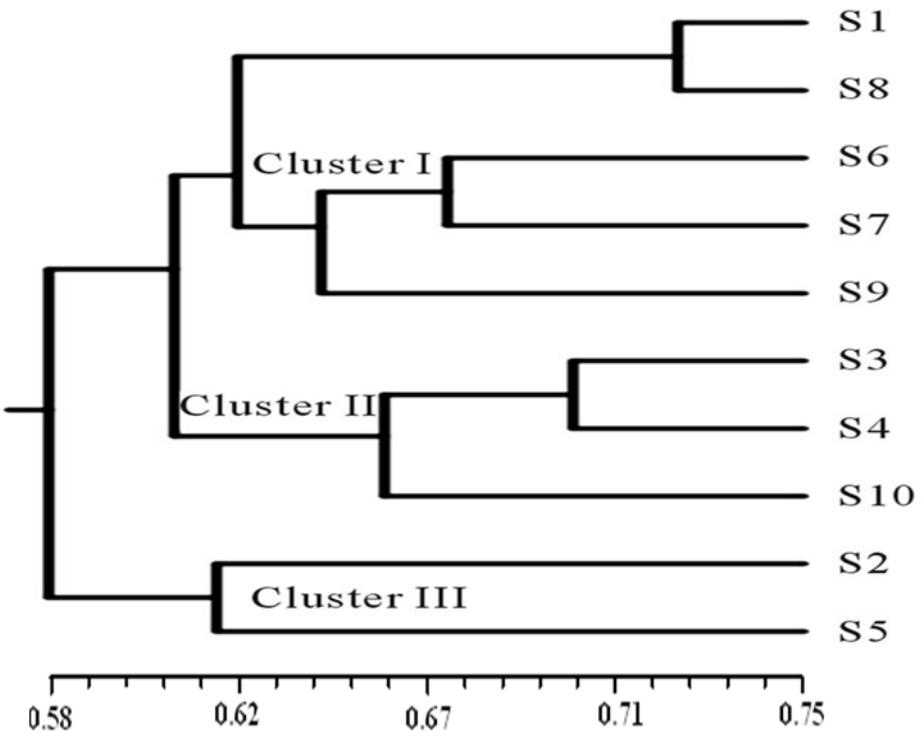

Figure 2. Dendrogram showing two clades of *C. occidentalis* accessions from 10 different localities.

S1- Parassala, S2- Kazhakuttam S3 -Amaravilla, S4- Neyyattinkara, S 5 -Attingal, S 6- Kattakada, S 7 -Aryanadu, S 8-Aruvikkara, S 9- Palode and S10 -Vembayam.

CONCLUSION

Taxonomic classification of plant species basically depends on the morphological characters, these features are not static and sometimes difficult to observe, so it is necessary to be supported by the molecular data. The present results of morphological and molecular data on *C. occidentalis* accessions demonstrate the variations of the plant collected from selected localities of Thiruvananthapuram. Although a good correlation observed between genetic and morphological data, future studies involving a large number of morphological traits with molecular markers should have important implications for germplasm management. The development of *C. occidentalis* with superior drug properties will be very important for promoting commercial production. RAPD makers have proved to be effective for characterizing the genetic basis for assessing genetic diversity and relatedness between accessions. The result of this study is of critical importance for breeding programs as well as informed and efficient management of germplasm collections.

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