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IDENTIFICATION OF CASTOR (*RICINUS COMMUNIS* L.) ECOTYPES THROUGH MOLECULAR CHARACTERIZATION IN THE SELECTED REGIONS OF THE WESTERN GHATS OF KARNATAKA, INDIA

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Abstract: Castor (*Ricinus communis* L.) being a perennial crop widely grown for oil seed production in tropical and sub-tropical regions of the world. Nevertheless, the leaf of castor serves as a primary food for the eri silkworm, *Samia cynthia ricini* Boisduval. Eri silkworm being a polyvoltine requires leaf throughout the year for its survival and cocoon production. Keeping this in view, an attempt has been made to identify (through molecular characterization) the best castor ecotype(s) found in different regions of Western Ghats of Karnataka, India for leaf production. The ecotypes were processed through DNA sequencing using ITS4 and ITS5 primers. The sequence results were authenticated through National Centre for Biotechnology Information by way of obtaining accession numbers (phylogenetic tree). Further, leaf samples were subjected to SDS-PAGE to know the variations existed among the ecotypes in protein profile. The results revealed that, ecotypes of different regions exhibits close relation among them and some marginal variations were evident in phylogenetic tree as well as in dendrogram. However, phylogenetic relationship of ecotypes in the major clade II and cluster III showed similar in both phylogeny and dendrogram for eight among 12 ecotypes representing different agro-ecological regions of Western Ghats of Karnataka. Further, five ecotypes showed close relationship in both phylogenetic as well as in cluster dendrogram, but in clades I and III, bootstrap values showed minor variation among the ecotypes representing different regions of the Western Ghats, whereas, in protein profile clusters I and II showed similarities between the ecotypes having genetic distance of 0.57. The maximum of 18 protein bands were found in KJ130046 ecotype, accordingly, minimum bands (10) were noticed in both KJ000404 and KJ000405 ecotypes.

Key Words: Ecotypes, Molecular Characterization, *Ricinus communis*, *Samia cynthia ricini*, Western Ghats.

INTRODUCTION

Castor (*Ricinus communis* L.) belongs to the family Euphorbiaceae and cultivated in wild form for oil seed production since ancient times at Gangetic plains and South Africa. Castor is monotypic and *R. communis* is only species with most polymorphic forms [1]. Castor has medicinal value and grows as perennial in tropical and sub-tropical regions of the world in varied types of soil and climatic conditions. The major castor growing countries in the world are India, China, Brazil, Russia, Thailand, Ethiopia and Philippines [2].

India is also an origin of castor which grows in natural condition and spread through semi wild and wild forms in diverse habitats like forest, sea coast, river bunds, railway tracks, garbage dumps, and waste land. India is in possession of 4373 castor accessions, of which 3416 accessions are being maintained by the Directorate of Oil seeds Research, Hyderabad and remaining 957 accessions were conserved by the National Bureau of Plant Genetic Resources (NBPGR), New Delhi [3]. In India, castor is found in wild condition in the states of Bihar, Uttar Pradesh and Madhya Pradesh with approximately 14 ft. tall and woody perennial type bearing big leaves [4].

Among the Euphorbiaceae family, castor is the only species which has lowest DNA c-value at which genome size of castor bean is around 350 mbp and has been sequenced and assembled in 4x draft using whole genome containing 31,221 proteins, although the function of most of these proteins remain unknown [5] [6]. The sequenced data of castor plant was obtained from various resources like National Centre for Biotechnology Information (NCBI) and J. Craig Venter Institute (JCVI). Molecular phylogenetic analyses indicate that *Ricinus* is closely related to *Sperkansia*, a genus native to china, and confirm that *Ricinus* is part of a natural lineage containing those genera in the tribe Acalyheae [7]. Sequence allows the identification of species from even a small processed material. In plants, the internal transcribed spacer (ITS) region of nuclear ribosomal cistron (18S-5.8S-26S) sequencing is the most commonly used sequence analysis for identification at the species level. Though castor has been mainly cultivated for its oil seed production, nevertheless, the leaves of which can be used for rearing the domesticated vanya silkworm (*Samia cynthia ricini* Boisduval) for cocoon production, as it serves as a primary food plant. In this backdrop, an attempt has been made to select the best ecotype(s) of castor (wild) in selected regions of the Western Ghats of Karnataka through molecular characterization.

MATERIALS AND METHODS

Study area

The study area includes five selected regions of Western Ghats of Karnataka comprising Heggada Devana Kote, Madikeri, Sakaleshpur, Shimoga and Sirsi along with Mysore for comparison (Figure 1 and Table 1).

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Figure 1: Maps showing selected regions of Western Ghats of Karnataka

Table 1: Elevation details of selected regions of Western Ghats of Karnataka

Sl. No.	Region	Latitude	Longitude	Altitude (MSL)
1.	HEGGADA DEVANA KOTE	12°05' N	76°19' E	694
2.	MADIKERI	12°26' N	75°47' E	970
3.	SAKALESHPUR	12°58' N	75°47' E	949
4.	SHIMOGA	12°56' N	75°38' E	569
5.	SIRSI	14°37' N	74°51' E	590
6.	MYSORE (CONTROL)	12°15' N	76°42' E	770

Two types of leaves (pink and green) based on their physical appearance were collected in each region of the study area were stored in -20°C. The castor leaf material was immersed in liquid nitrogen and crushed into fine powder using autoclaved mortar and pestle. The samples were processed for identification of ecotypes through molecular characterization based on DNA sequencing using DNA extraction kit (Himedia).

PCR Amplification

The complete ITS region (ITS1, the 5.8S gene and ITS 2) was amplified using primers ITS-4 and ITS-5 [8] and about 25 µl of reaction mixture containing 100 µM of deoxynucleoside triphosphate, 0.1 µM each primer, 1 x PCR buffer, 2 µl of template DNA and I unit of Taq polymerase. The thermal cycler reaction was involved in initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94° C for 45 seconds, annealing at 50° C for 45 seconds, extension at 72° C for 40 seconds and final extension at 72°C for 7 min. Following amplification, 5 µl of the reaction mixture was run on a 1.5% agarose gel in 0.5× TAE buffer to determine an appropriate sized product. PCR products or amplicons were visualized using ethidium bromide under UV light.

Sequencing was performed in an automated ABI 3100 Genetic Analyser (Applied Biosystems, CF, USA) by Amnion Biosciences (Bangalore, India). Forward and reverse sequencing were done individually with ITS4 and ITS5 primers, respectively. Sequence results were submitted to Gen Bank, National Centre for Biotechnology Information (NCBI), Bethesda MD, USA. Sequence of ITS regions was analyzed using BLAST tool in the NCBI website and sequences were deposited in the NCBI nucleotide sequence database, Gen Bank [9].

Sequencing results were processed using Bio Edit software [10]. The processed sequences were subjected to BLAST search in webpage (www.ncbi.nlm.nih.gov) for better identification of sequence at species level and for phylogenetic inference. Sequences were then aligned and compared with other similar sequences retrieved from Gen Bank using online Clustalw software and Bio Edit software, respectively [11] [12]. Alignments were manually edited where necessary and phylogenetic analyses performed by using maximum likelihood (ML) or neighbour joining method in MEGA Version 6.0 (software package) combined with bootstrap analysis with 1,000 replications to assess intra-specific variation among the sequences [13].

Gel electrophoresis

One gram of fresh leaf samples of each ecotype was homogenate with phosphate buffer (pH 7.0) using pre-chilled mortar and pestle centrifuged at 4° C at 10,000 rpm for 10 min and the supernatant was collected and used for protein profiling through Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS - PAGE) [14]. In the process of running SDS – PAGE, glass plates were assembled by preparing approximate volume of acrylamide (30% acrylamide and 0.8 % bis-acrylamide) and made upto 100 ml with distilled water. The separating gel (10%) was prepared by 3.34 ml of acrylamide, 2.5 ml of Tris HCl (1.5M tris HCl buffer pH 8.8), 0.1 ml of 10% SDS, 10% of ammonium per sulphate (APS) and 20µl of TEMED were mixed with 3.8 ml of distilled water and poured the gel solution in between the glass plates at about 4cm from the top of the plate. Later, about 2-3 drops of isobutanol was added from the top to avoid air bubbles in the gel and leave it for 30 min. Staking gel (5%) was prepared by adding 1.75 ml of acrylamide solution, 2.25 ml of tris HCl (0.5m tris buffer of pH 6.8), 0.1 ml of 10% SDS, 0.1 ml APS, 2.89 ml of distilled water and 10 µl of TEMED were added and mixed well. The mixture was poured to slab without entering air bubbles and comb was inserted immediately to form 1 mm thickness of gel and left for 30min. Comb was removed by adding sample buffer and leaf samples at 1:1 ratio and boiled for 5 min and later allowed for cooling. Equal proportion of protein samples were loaded to each well using medium range protein marker (Genei). The gel was run using tank buffer with 100 volts for about three hours, later the gel was stained with coomassie brilliant blue (R250) for 20 hours and de-stained.

Further, the gel was scanned using Alpha Innotech Gel Documentation Unit and analyzed for the banding pattern of ecotypes.

RESULTS AND DISCUSSION

The results of the study revealed that the 12 castor ecotypes identified from selected regions of Western Ghats of Karnataka recorded a size of 700 base pairs in length as evidenced through the PCR products or amplicons (Fig. 2). Twelve ecotypes were amplified and sequenced nucleotides were submitted to multiple sequenced alignments using Clustalw (Fig. 3). The accession numbers were obtained from NCBI for 12 ecotypes *viz.*, KJ000402, KJ000403, KJ000404, KJ000405, KJ000406, KJ000407, KJ130043, KJ130044, KJ130045, KJ130046, KJ130047 and KJ130048 (Table 2).

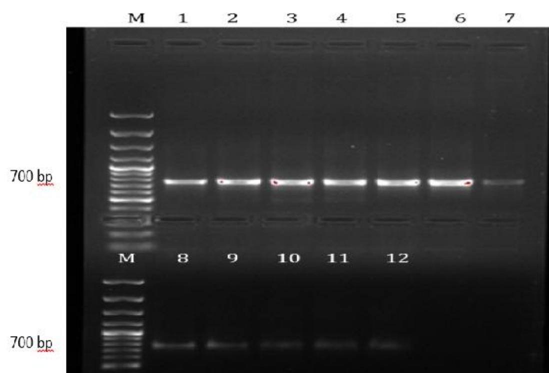

Figure 2: Agarose gel electrophoresis showing expected amplicon size around ~700 base M - Marker DNA (1Kb Ladder), 1 - KJ000402, 2 - KJ000403, 3 – KJ000404, 4 – KJ000405, 5 – KJ000406, 6 - KJ000407, 7 – KJ130043, 8 – KJ130044, 9 – KJ130045, 10 – KJ130046, 11 – KJ130047 and 12 – KJ130048

Table 2: Accession numbers of identified ecotypes with size of PCR product in selected regions of Western Ghats of Karnataka

Code	Region	Ecotype	Accession no.	Size of PCR product (bp)
S-1	HEGGADADEVANA KOTE	PINK	KJ000402	705
S-2	HEGGADADEVANA KOTE	GREEN	KJ000403	698
S-3	MADIKERI	PINK	KJ000404	683
S-4	MADIKERI	GREEN	KJ000405	629
S-5	SAKALESHPUR	PINK	KJ000406	683
S-6	SAKALESHPUR	GREEN	KJ000407	690
S-7	SHIMOGA	PINK	KJ130043	672
S-8	SHIMOGA	GREEN	KJ130044	682
S-9	SIRSI	PINK	KJ130045	686
S-10	SIRSI	GREEN	KJ130046	685
S-11	MYSORE	PINK	KJ130047	684
S-12	MYSORE	GREEN	KJ130048	681

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KJ130047    ------------------GACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 42
KJ000402    ---------------CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 45
KJ130043    ---------------------------TCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 33
KJ000405    ------------------------------------------------------------
KJ000404    ----------------GTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 44
KJ130046    ---------------CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 45
KJ000406    --------------TCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 46
KJ000407    -------AAGTAAAAAGT-CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 56
KJ130045    ---------AGTAAAAAGT-CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 55
KJ000403    GTAAAGTAAAAAAAGCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTGTCG 60
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KJ000406    AAACCTGCCCTGCAGAACGACCCCGCGAACATGTTTGCTTATTGCAAGGGGGAGCGGGGG 106
KJ000407    AAACCTGCCCTGCAGAACGACCCCGCGAACATGTTTGCTTATTGCAAGGGGGAGCGGGGG 116
KJ130045    AAACCTGCCCTGCAGAACGACCCCGCGAACATGTTTGCTTATTGCAAGGGGGAGCGGGGG 115
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KJ130043    TCGCCATGGCCCCAGTCCCCC-GATGTCGGCGAGAGGGGTGGGGTGGGGCTTTGCCCCCT 152
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KJ000406    TCGCCATGGCCCCAGTCCCCC-GATGTCGGCGAGAGGGGTGGGGTGGGGCTTTGCCCCCT 165
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KJ130045    GATGCTGCC-GTAAAGGGCATGCTCCAACTGCG--------------------------- 686
KJ000403    GATGCTGCC-GTAAAGGGCATGCTCCAACTGCGACCCCAG-------------------- 698
KJ130048    GATGCTGCC-GTAAAGGGCATGCTCCAACTGCGACC------------------------ 681
            ********* ***********************
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KJ130044	--
KJ130047	--
KJ000402	TT 705
KJ130043	--
KJ000405	--
KJ000404	--
KJ130046	--
KJ000406	--
KJ000407	--
KJ130045	--
KJ000403	--
KJ130048	--

Figure 3: The sequenced alignment of identified castor ecotypes using Clustalw

The castor ecotypes were initially identified from selected regions of Western Ghats of Karnataka based on the morphological features and all the ecotypes exhibited differences in leaf shape, stem colour and seeds. The classification and description of germplasm collections were generally identified by morphological characterizations [15]. However, phenotypic variations were evident in respect of leaf shape and size, stem colour and seed texture in different cultivars. It is an important to characterize the genetic diversity in present across *R. Communis* in germplasm from different geographical regions developed a genotyping links in castor bean from the particular sources in the geographical area [16].

Gel electrophoresis

The castor leaf samples from 12 ecotypes were collected from selected regions of Western Ghats of Karnataka was subjected to SDS PAGE to understand the expression of protein profile. Protein profile through electrophoresis is a tool for identification of genetic diversity through SDS PAGE with respect to environmental fluctuations is highly independent from seed storage proteins and consider as a most reliable technique [17]. The electrophoretic protein profile was revealed on the basis of protein marker with medium range of molecular weight (19.1 to 97.4 k Da) and genetic differences in each ecotype were ascertained through dendrogram. The expression of protein bands in castor ecotypes were maximum (18 bands) in KJ130046, however, minimum (10 bands) were observed in KJ000404 and KJ000405. Accordingly, protein bands of 17, 16, 16, 15, 15, 13, 13, 12 and 11 were recorded in other ecotypes namely KJ130047, KJ130048, KJ130045, KJ130044, KJ000406, KJ000402, KJ000403, KJ130043 and KJ000407, respectively (Fig. 4).

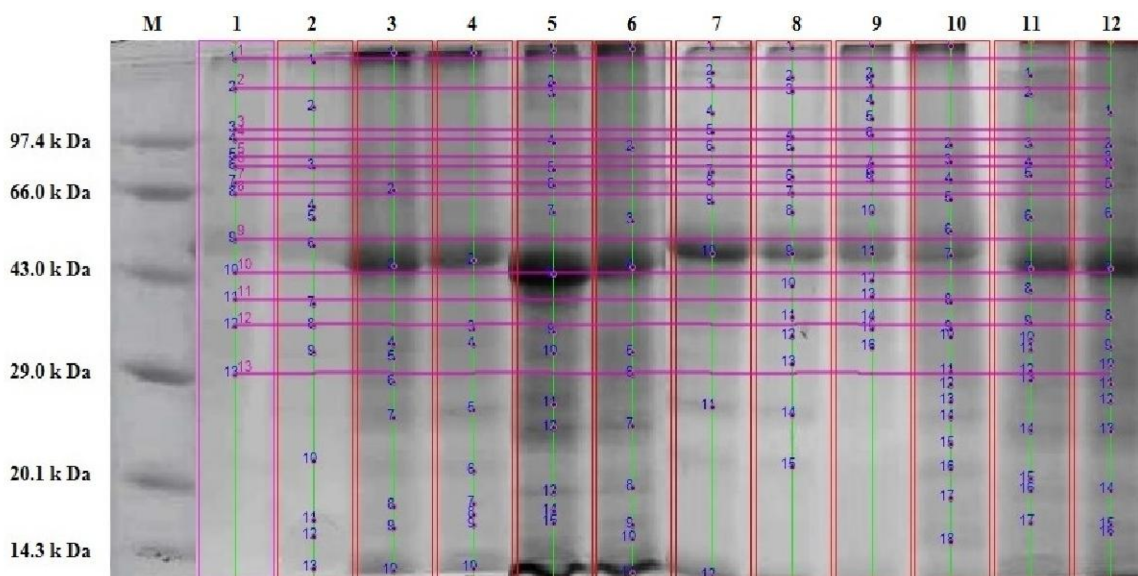

Figure 4: Protein profile of castor ecotypes identified from selected regions of Western Ghats of Karnataka

M - Marker Medium range, 1 - KJ000402, 2 - KJ000403, 3 – KJ000404, 4 – KJ000405, 5 – KJ000406, 6 - KJ000407, 7 – KJ130043, 8 – KJ130044, 9 – KJ130045, 10 – KJ130046, 11 – KJ130047 and 12 – KJ130048

The phylogenetic analysis of castor ecotypes of the Western Ghats of Karnataka was performed using maximum likelihood tree with the help of MEGA 6.06 [10] [11]. The molecular marker has been used for identification of biological organisms through molecular techniques i.e., SDS-PAGE and was found most reliable. Further, it should be common for identification of species, varieties and ecotypes among the genetically diversified species [18] [19]. However, the DNA sequences of single gene generally do not provide adequate phylogenetic resolution for population level in plants [20]. Further, 12 ecotypes were distributed among 3 clades in 3 clusters of polygenetic tree (Fig. 5) and SDS PAGE (Fig. 6), respectively. Among the *R. communis,* genetic variations were grouped into two clades and it reflects on all castor cultivars derived from limited genetic pool of old world plant from Asia or Africa [16].

Figure 5: Phylogenetic tree represents castor ecotypes using maximum likelihood

Phylogenetic analysis as well as dendrogram from protein electrophoresis exhibited similar clustering with minor variations. The major clade II and cluster III from phylogeny and dendrogram shares eight ecotypes of castor among 12 ecotypes. The ecotypes bearing accession numbers KJ130046, KJ000404, KJ000403, KJ000406 and KJ130045 were found similar and represent close relationship in both phylogeny as well as in cluster dendrogram with well supported bootstrap values and genetic distance, respectively, whereas, KJ130048, KJ130047 and KJ000402 ecotypes represents different clades as well as in cluster. Distance analysis of the data using neighbour-joining yields highly resolved tree as a consequence of the algorithm but has virtually no bootstrap support for any of the group and were extremely small divergences between castor varieties. Consequently, the data suggested that, it cannot be relied upon phylogenetic analysis of preproricin sequences to know much about the relationship between varieties of castor bean plants or their geographical origin [21].

The clade-I (KJ000405 and KJ000407) and clade-III (KJ130043 and KJ130044) consists of two each ecotype in phylogeny with different clades with slight variation genetically. Protein electrophoresis is widely used among the biochemical techniques with SDS-PAGE to identify and describe the genetic structure of crop germplasm [22] [23] [24]. Whereas, in protein profile, cluster-I (KJ130043 and KJ000402) and cluster-II (KJ130048 and KJ130047) showed the genetic difference within the cluster of 0.57 and there is no genetic difference between the clusters.

Figure 6: Dendrogram represents clusters among the castor ecotypes

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