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RESEARCH ARTICLE

DNA BARCODING AND MOLECULAR STUDIES IN GYMNEMA SPECIES

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ABSTRACT

Gymnema spp studied in the present research are *G. latifolium, G. elegans, G. hirsutum* and *G. sylvestere*. They are important woody climber of family Asclepiadaceae, grows well in tropical and subtropical forests of Indian subcontinent. The plants were used in folk and Ayurvedic system of medicines for the treatment of diabetes, additionally, the plants also possesses antimicrobial, hepato protective and sweet suppressing activities and also used for the treatment of asthma, inflammations, and snake bite. The various reports on its multiple uses attracted attention fortilization of the plant for the major bioactive principle gymnemic acid. Therefore, an initiative has been taken to estimate the level of diversity and genetic relationship among the selected four *Gymnema* spp. 10 random decamer primers were used for RAPD analysis and Rbcl region of ribosome were also used. Among the 10 primers used only 5 primers produced reproducible and consistent banding pattern, those are OPA-17, OPE-14, OPE-15, OPE-17 and OP0-16. In that OPE-14 produced height 24 bands. And OPA-17 produced 16 bands and in that 14 were polymorphic bands and the percentage of polymorphism was 87.5%.PCR success rate for rbcl loci was 100% and sequence success rate was also 100% success. BLAST results revealed that the three species sequences for rbcl loci was 98% similar. The dendrogram revealed the phylogenitic order of the species.

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INTRODUCTION

DNA barcoding is a technique that is used to identify the species based on species-specific differences in short regions of their DNA (Hebert *et al*., 2003). DNA barcoding uses state-of-the-art biotechnology to identify plant species in a rapid, accurate, and cost-effective manner. This technique is not restricted by morphological characteristics, physiological conditions, and allows species identification without specialist taxonomic knowledge. DNA barcoding uses the data of one or a couple of regions in the genome to recognize all the species in a particular class (Lahaye, 2008). This technique opened up new doors for DNA-based examinations extending from group phylogenetics to environmental genomics (Webb *et al.,* 2002; van Straalen *et al*., 2006). It has been reported that DNA barcoding is not only used to build phylogenetic trees, but to provide rapid and accurate identification of unknown organisms. This is an efficient tool to identify the species because levels of divergence among individuals of the same species are usually much lower than the closely-related species that exhibit a "barcoding gap" between inter- versus intraspecific divergences (Armstrong 2005). In order to make DNA barcoding information universally and publically accessible, new databases have been made available online.

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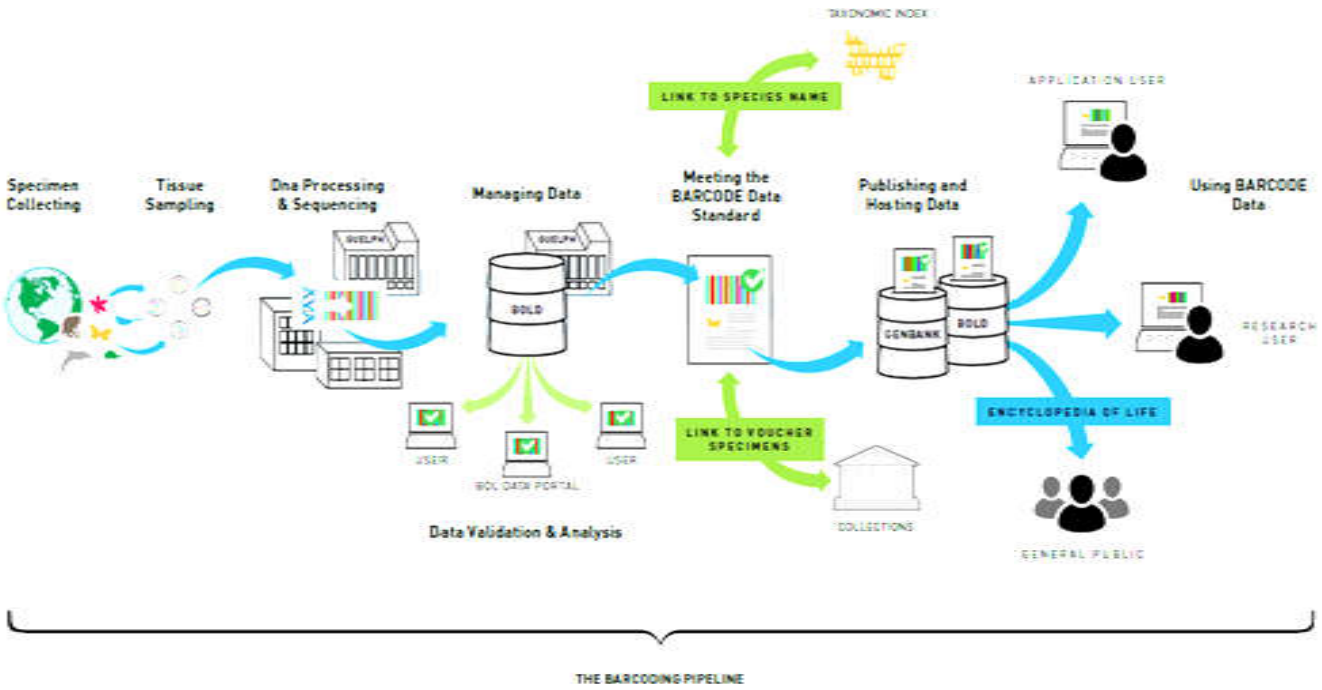
Rapid progress in DNA sequencing and computational technologies made CBOL to build a universal organization for living beings inventory: the Barcode of Life Database (BOLD) system. BOLD is created and maintained by the University of Guelph in Ontario, Canada (http://www.boldsystems.org) (CBOL Plant Working Group 2009). It facilitates researchers to collect, manage, and analyze DNA barcode data. BOLD will provide a DNA barcode to clearly identify the unknown specimens by facilitating accurate query assignments and by comparing the data that are obtained from geographically dispersed institutions. BOLD could serve as the universal starting point for species identification, which would convey to the users to refer the specialized databases (eg, endangered species, pathogenic strains, disease vector species,) (Ratnasingham and Hebert 2007.

MATERIALS AND METHODS

Plant materials: The fresh leaves have to be taken for DNA extraction.

DNA Extraction: The DNA isolation procedure here used is a modification of the Carlson/Yoon method (Yoon *et al.,* 1991), and is presented below: The sample fragments will transfer to a mortar and ground with grinding sand until homogenized. No more than 500 µl of the ground material will transferred to a 2 ml tube.



To this 750 µl of CTAB (hexadecyl trimethyl ammonium bromide) will be added and 20 µl mercaptoethanol. The tube was incubated at 65°C for 45 min, and mixed intermittently by inverting. Subsequently, 750 µl of chloroform/isoamylalchohol (24:1) will be added and the tube put horizontally in a shaker and shaken at 100 rpm for 30 min. The tube will then centrifuged at 12000 x g for 10 min. The upper aqueous phase (containing the DNA) will be transferred to a new 1.5 ml tube. This chloroform/isoamylalchohol extraction step have to be repeated and the tube centrifuged for 5 min. The aqueous phase now have to be again transferred to a new 1.5 ml tube. The DNA will be precipitated with 0.1 vol. of 3M NaAc (pH 4.6) and 2 vol. of 95% EtOH and incubated at -20°C for 1 hour or overnight. The precipitate will centrifuged at 12000 x g for 10 min, after which the liquid will discard (the DNA pellet remaining at the bottom of the tube), and 750 µl of 70% EtOH will be added. Now this will be mixed and centrifuged for 5 min. Again, the ethanol will poured off, the tube centrifuged for a few seconds, and the remaining liquid was removed with a pipette. The tube will placed horizontally in the fume hood (with the cap open) for 30 min. 100 µl of EB buffer will be added.

PCR amplification and sequencing

The primers used for the amplification of different loci are listed in Table 1. Primers for the loci from the chloroplast genome were taken from the Kew website (http://www.kew.org/barcoding/protocols.html). The corresponding modified sequences were then taken as the primers for amplification of the respective loci. A total of 25 µl reaction mixture contained 1 unit of Pfu DNA polymerase, 2 µl of 10X PCR buffer with 20 mM MgSO4, 2 µl of 2mM dNTPs, 2 µl of each primer (10 lM) and 20–30 ng of template DNA. Thermal cycle followed for ITS was as per Tsai *et al*. (2004). For loci from the chloroplast genome, the thermal cycle used was as follows: one cycle of DNA denaturation at 95 ^{O}C for 5 min, followed by 35 cycles of 30 s at 95 ^{O}C, 45 s at 50 ^{O}C and 1 min at 72 ^{O}C, with a final extension of 5 min at 72 ^{O}C. The PCR products were separated by electrophoresis in 1% TBE agarose gels, containing EtBr (0.5 lg / ml), and visualized on a UV transilluminator.

All single-band amplicons were cleaned using Exo- SAP method. The reaction mixture consisted of 0.5 µl of Exonuclease I (10 U; Fermentas Inc.), 1 µl of Shrimp Alkaline Phosphatase (1 U; Fermentas Inc.), 0.5 µl of MQ and 8 µl of PCR product. This was incubated at 37 ^{O}C for 15 min, 85 ^{O}C for 15 min and finally held at 4 ^{O}C in a thermal cycler. Where multiple bands were obtained after amplification, only the band having molecular weight nearest to that of the targeted region was purified using a column-based DNA purification kit, GeneJET Gel Extraction kit (Fermentas Inc.), according to the manufacturer's instructions. The final PCR products in both cases were subjected to Sanger's di-deoxy sequencing, in both forward and reverse directions, using BigDye terminator v3.1 cycle sequencing kit on ABI Prism 3700 DNA Analyzer (Applied Biosystems Inc., USA) as per manufacturer's instructions.

RAPD analysis

After estimating the quantity of the DNA, the samples were adjusted to a concentration of 50 ng/µL. A total of 20 RAPD primers were selected and used for amplification (Table 1). The RAPD reactions were performed with the following concentrations: genomic DNA (50 ng), Buffer 1X, $MgCl_2$ (2mM), dNTPs (0.1mM), primer (0.4 µM), Taq DNA polymerase (1 unit), and distilled water in a final volume of 25 µl. Amplification was performed in a Biorad Mini thermal cycler with the following program: one cycle of 95 ºC for 5 minutes, forty cycles of 95 ºC for 30 seconds, 37 ºC for 1 min and 72 ºC for 2 minute, followed by a final cycle of 5 minutes at 72 ºC. The amplification products were subjected to electrophoresis on a 2% agarose gel. The gel was stained with 5mg/ml ethidium bromide solution. The amplified fragments were visualized under UV light and photographed using a Biorad XR® digital photo documentation system.

Data analysis

The electropherograms obtained after sequencing were base-called using PHRED, and the forward and reverse sequences were trimmed and assembled using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). The sequences have been

submitted to the NCBI GenBank. The inter- and intra-specific distances were calculated using K2P (Kimura 2 parameter) model as recommended by the Consortium of Barcode of Life (CBOL, http:// www.barcoding.si.edu/protocols.html) using MEGA 4.0 software.

4 species were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of a particular band. The data was analyzed using NTSYS software package (Rohlf, 1990). The SIMQUAL programme was used to calculate Jaccard's

Table 1. Primers used for amplification of candidate DNA barcodes S. no. Locus Primer name Primer sequence

S.no	Locus	Primer Name	Primer Sequence
1	matK	Rbcl-f	5'- ATGTCACCACAAACAGAAACTAAAGC-3'
		Rbcl-r	5'- CTTCGGCACAAAATAAGAAACGATCTC-3'
5	RAPD	OPA-04	AATCGGGCTG
		OPA-08	GTGACGTAGG
		OPA-11	CAATCGCCGT
		OPA 17	GACCGCTTGT
		OPA 16	AGCCAGCGAA
		OPE-06	AAGACCCCTC
		OPE-09	CTTCACCCGA
		OPE 14	TGCGGCTGAG
		OPE 15	ACGCACAACC
		OPE 17	CTACTGCCGT

Table 2. Table showing total number of bands and number of polymorphic bands produced from the selected five RAPD primers

S.No	Primer	Total number of bands	Total number of polymorphic bands	Percentage of polymorphism
1	OPA-17	16	14	87.5
2	OPE-14	24	16	66.6
3	OPE-15	11	7	63.6
4	OPE-17	12	9	75
5	OPO-16	11	6	54.5

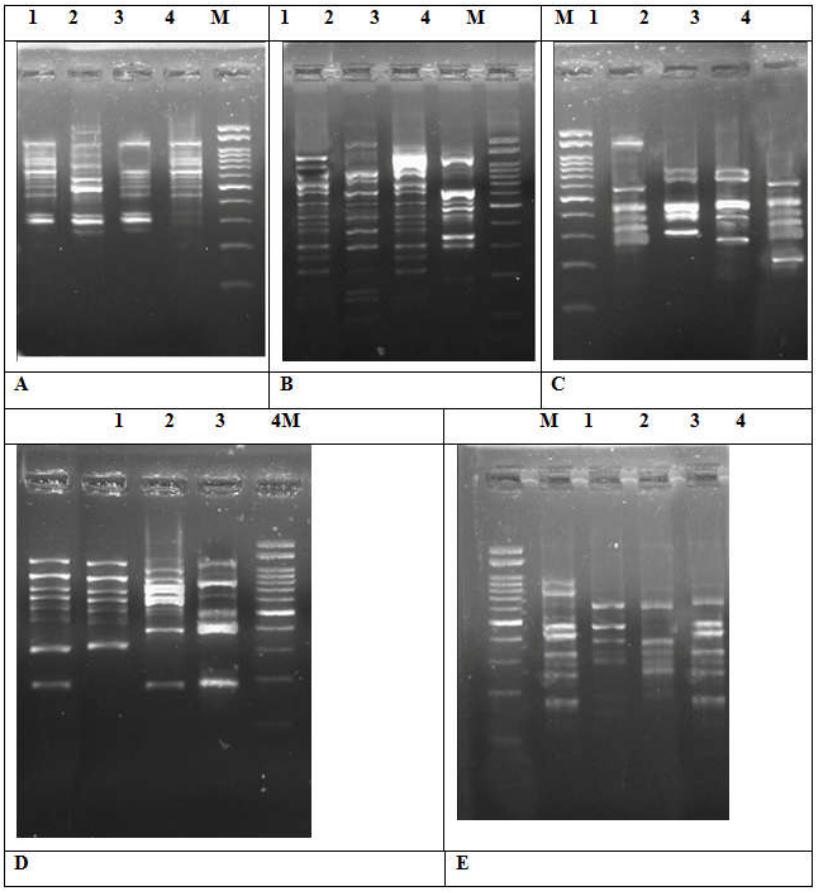


Figure 1. Figure showing RAPD banding pattern of three *Gymnema* spp. with different primers; A-OPA-17; B-OPE-14; C-OPE-15; D-OPE-17 and E- OPO-16. Lane 1- *G. latifolium;* Lane 2- *G. elegans*Lane 3- *G. hirsutum;* lane 4- *G. sylvestere* and Lane-M 100 base pairs marker

The species identification success rate was calculated using genetic distance and BLAST methods. Besides, rbcl sequences, affording 100% species resolution based on K2P method, were blasted on NCBI BLAST for checking their homology with other available sequences. For RAPD Amplification profiles of

coefficient. Clustering was done using Sequential Agglomerative Heirarchial Nested Clustering (SAHN) routine and a dendrogram constructed using UPGMA(Sneath and Sokal, 1973) with NTSYS package.

RESULTS

Among the 10 primers used only 5 primers produced reproducible and consistent banding pattern, those are OPA-17, OPE-14, OPE-15, OPE-17 and OP0-16. In that OPE-14 produced height 24 bands. And OPA-17 produced 16 bands and in that 14 were polymorphic bands and the percentage of polymorphism was 87.5%. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.49385447 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of band substitutions per site. The analysis involved 3 species of *Gymnema*. All positions containing gaps and missing data were eliminated. There were a total of 68 positions in the final dataset of five RAPD primers.

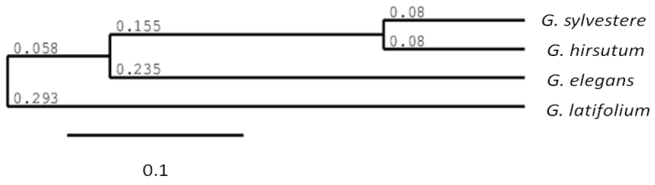


Figure 2. Dendrogram showing Evolutionary relationships between three species of *Gymnema*

rbcl Sequences of three *Gymnema spp*

RESULTS AND DISCUSSION

PCR success rate for rbcl loci was 100% and sequence success rate was also 100% success. BLAST results revealed that the three species sequences for rbcl loci was 98% similar. The dendrogram revealed the phylogenitic order of the species.

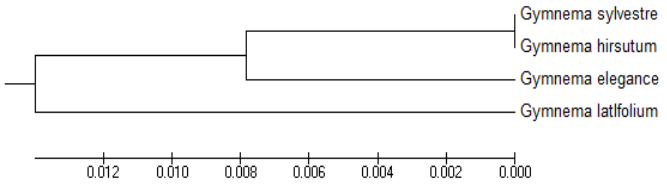


Figure. Dendrogram showing Evolutionary relationships between four species of *Gymnema* using rbcl sequences

The evolutionary history was inferred using the UPGMA method [1]. The optimal tree with the sum of branch length = 0.03582612 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 644 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [3].

>*Gymnema* sylvestre

TAAAGCAAGTGTTGGATTCAAAGCCGGTGTTAAAGA
GTACAAATTGACTTATTATACTCCTGAATACGAAACA
AAAGATACTGATATCTTGGCAGCATTCCGAGTAACTC
CTCAACCCGGAGTTCCACCCGAAGAAGCAGGGGCCG
CGGTAGCTGCCGAATCTTCTACTGGTACATGGACAAC
TGTTTGGACCGATGGACTTACCAGCCTTGATCGTTAC
AAAGGGCGATGCTACCATATCGAGCCCGTTCCTGGA
GAAGAAGATCAATTTATTGCTTATGTAGCTTACCCTT
TAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGCT
TACTTCCATTGTAGGTAATGTATTTGGGTTCAAAGCC
CTACGCGCTCTACGTTTGGAAGATTTGCGAATCCCTA
CGGCTTATATTAAAACCTTCCAAGGCCCACCGCATGG
CATCCAGGTTGAGAGAGATAAATTGAACAAATATGG
TCGTCCCCTGTTGGGATGTACTATTAAACCAAAATTG
GGGTTATCAGCTAAAAACTACGGTAGGGCGGTTTATG
AATGTCTTCGTGGTGGACTTGATTTTACCAAAGATGA
TGAAAACGTGAACTCCCAACCGTTTATGCGTTGGAGA
GATCGTTTCTTGTTTTGTGCCGAAGCAATTTTTAAATC
ACAGGCTGAAACTGGCG

>*GymnemalatIfolium*

AGGTGGGATTAAGGGGTGGCTAAGGGTTTACAAATT
GACTTATTATACTCCTGAATACGAAACAAAAGATACT
GATATCTTGGCAGCATTCCGAGTAACTCCTCAACCCG
GAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAGCTG
CCGAATCTTCTACTGGTACATGGACAACTGTTTGGAC
CGATGGACTTACCAGCCTTGATCGTTACAAAGGGCGA
TGCTACCATATCGAGCCCGTTCCTGGAGAAGAAGATC
AATTTATTGCTTATGTAGCTTACCCTTTAGACCTTTTT
GAAGAAGGTTCTGTTACTAACATGCTTACTTCCATTG
TAGGTAATGTATTTGGGTTCAAAGCCCTACGCGCTCT
ACGTTTGGAAGATTTGCGAATCCCTACCGCTTATATT
AAAACCTTCCAAGGCCCACCGCATGGCACCCAGGTT
GAGAGAGATAAATTGAACAAATATGGTCGTCCCCTG
TTGGGATGTACTATTAAACCAAAATTGGGGTTATCAG
CTAAAAACTACGGTAGGGCGGTTTATGAATGTCTTCG
TGGTGGACTTGATTTTACCAAAGATGATGAAAACGTG
AACTCCCACCCGTTTATGCGTTGGAGAGATCGTTTCT
TGTTTTGTGCCGAAGCACTT

> *Gymnema elegance*

AAGGGTGGAATTCAGGGCGGGTGTTAGAGAGTACAA
ATTGACTTATTATACTCCTGAATACCAAACAAAAGAT
ACTGATATCTTGGCAGCATTCCTAGTAACTCCTCAAC
CCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAG
CTGCCGAATCTTCTACTGGTACATGGACAACTGTTTG
GACCGATGGACTTACCAGCCTTGATCGTTACAAAGGG
CGATGCTACCATATCGAGCCCGTTCCTGGAGAAGAA
GATCAATTTATTGCTTATGTAGCTTACCCTTTAGACCT
TTTTGAAGAAGGTTCTGTTACTAACATGCTTACTTCC
ATTGTAGGTAATGTATTTGGGTTCAAAGCCCTACGCG
CTCTACGTTTGGAAGATTTGTGAATCCCTACGGCTTA
TATTAAAACCTTCCAAGGCCCACCGCATGGCATCCAG
GTTGAGAGAGATAAATTGAACAAATATGGTCGTCCC
CTGTTGGGATGTACTATTAAACCAAAATTGGGGTTAT
CAGCTAAAAACTACGGTAGGGCGGTTTATGAATGTCT
TCGTGGTGGACTTGATTTTACCAAAGATGATGAAAAC
GTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTT
TCTTGTTTTGTGCCGAAGCAA

> *Gymnema hirsutum*

AAGTGTTGGATTCAAAGCCGGTGTTAAAGAGTACAA
ATTGACTTATTATACTCCTGAATACGAAACAAAAGAT
ACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAAC

CCGGAGTTCCACCCGAAGAAGCAGGGGCCGCGGTAG
CTGCCGAATCTTCTACTGGTACATGGACAACTGTTTG
GACCGATGGACTTACCAGCCTTGATCGTTACAAAGGG
CGATGCTACCATATCGAGCCCGTTCCTGGAGAAGAA
GATCAATTTATTGCTTATGTAGCTTACCCTTTAGACCT
TTTTGAAGAAGGTTCTGTTACTAACATGCTTACTTCC
ATTGTAGGTAATGTATTTGGGTTCAAAGCCCTACGCG
CTCTACGTTTGGAAGATTTGCGAATCCCTACGGCTTA
TATTAAAACCTTCCAAGGCCCACCGCATGGCATCCAG
GTTGAGAGAGATAAATTGAACAAATATGGTCGTCCC
CTGTTGGGATGTACTATTAAACCAAAATTGGGGTTAT
CAGCTAAAAACTACGGTAGGGCGGTTTATGAATGTCT
TCGTGGTGGACTTGATTTTACCAAAGATGATGAAAAC
GTGAACTCCCAACCGTTTATGCGTTGGAGAGATCGTT
TCTTGTTTTGTGCCGAAGCAATTTTTAAATCACAGGC
TGAAACTGGCGAAATCAAAGGACATTACTTGAATGC
TAC

Pair wise alignment of rbcl sequences from three *Gymnema spp.*

Maximum Likelihood Estimate of Transition/Transversion Bias

The estimated Transition/Transversion bias (*R*) is 0.73. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model [Kimura 1980]. The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -1038.836. The analysis involved 3 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

Alignment: Untitled1

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
G.sylvestr      TAAAGCAAGT GTTGGATTCA AAGCCGGTGT TAAAGAGTAC AAATTGACTT
G.latIfoli      --------AG GTGGGATTAA GGGGTGGC-T AAGGGTTTAC AAATTGACTT
G.elegans       ------AAGG GTGGAATTCA GGGCGGGTGT TAGAGAGTAC AAATTGACTT
G.hirsutum      ------AAGT GTTGGATTCA AAGCCGGTGT TAAAGAGTAC AAATTGACTT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
G.sylvestr      ATTATACTCC TGAATACGAA ACAAAAGATA CTGATATCTT GGCAGCATTC
G.latIfoli      ATTATACTCC TGAATACGAA ACAAAAGATA CTGATATCTT GGCAGCATTC
G.elegans       ATTATACTCC TGAATACCAA ACAAAAGATA CTGATATCTT GGCAGCATTC
G.hirsutum      ATTATACTCC TGAATACGAA ACAAAAGATA CTGATATCTT GGCAGCATTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
G.sylvestr      CGAGTAACTC CTCAACCCGG AGTTCCACCC GAAGAAGCAG GGGCCGCGGT
G.latIfoli      CGAGTAACTC CTCAACCCGG AGTTCCACCC GAAGAAGCAG GGGCCGCGGT
G.elegans       CTAGTAACTC CTCAACCCGG AGTTCCACCC GAAGAAGCAG GGGCCGCGGT
G.hirsutum      CGAGTAACTC CTCAACCCGG AGTTCCACCC GAAGAAGCAG GGGCCGCGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
G.sylvestr      AGCTGCCGAA TCTTCTACTG GTACATGGAC AACTGTTTGG ACCGATGGAC
G.latIfoli      AGCTGCCGAA TCTTCTACTG GTACATGGAC AACTGTTTGG ACCGATGGAC
G.elegans       AGCTGCCGAA TCTTCTACTG GTACATGGAC AACTGTTTGG ACCGATGGAC
G.hirsutum      AGCTGCCGAA TCTTCTACTG GTACATGGAC AACTGTTTGG ACCGATGGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
G.sylvestr      TTACCAGCCT TGATCGTTAC AAAGGGCGAT GCTACCATAT CGAGCCCGTT
G.latIfoli      TTACCAGCCT TGATCGTTAC AAAGGGCGAT GCTACCATAT CGAGCCCGTT
G.elegans       TTACCAGCCT TGATCGTTAC AAAGGGCGAT GCTACCATAT CGAGCCCGTT
G.hirsutum      TTACCAGCCT TGATCGTTAC AAAGGGCGAT GCTACCATAT CGAGCCCGTT
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
G.sylvestr      CCTGGAGAAG AAGATCAATT TATTGCTTAT GTAGCTTACC CTTTAGACCT
G.latIfoli      CCTGGAGAAG AAGATCAATT TATTGCTTAT GTAGCTTACC CTTTAGACCT
G.elegans       CCTGGAGAAG AAGATCAATT TATTGCTTAT GTAGCTTACC CTTTAGACCT
G.hirsutum      CCTGGAGAAG AAGATCAATT TATTGCTTAT GTAGCTTACC CTTTAGACCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
G.sylvestr      TTTTGAAGAA GGTTCTGTTA CTAACATGCT TACTTCCATT GTAGGTAATG
G.latIfoli      TTTTGAAGAA GGTTCTGTTA CTAACATGCT TACTTCCATT GTAGGTAATG
G.elegans       TTTTGAAGAA GGTTCTGTTA CTAACATGCT TACTTCCATT GTAGGTAATG
G.hirsutum      TTTTGAAGAA GGTTCTGTTA CTAACATGCT TACTTCCATT GTAGGTAATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
G.sylvestr      TATTTGGGTT CAAAGCCCTA CGCGCTCTAC GTTTGGAAGA TTTGCGAATC
G.latIfoli      TATTTGGGTT CAAAGCCCTA CGCGCTCTAC GTTTGGAAGA TTTGCGAATC
G.elegans       TATTTGGGTT CAAAGCCCTA CGCGCTCTAC GTTTGGAAGA TTTGTGAATC
G.hirsutum      TATTTGGGTT CAAAGCCCTA CGCGCTCTAC GTTTGGAAGA TTTGCGAATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
G.sylvestr      CCTACGGCTT ATATTAAAAC CTTCCAAGGC CCACCGCATG GCATCCAGGT
G.latIfoli      CCTACCGCTT ATATTAAAAC CTTCCAAGGC CCACCGCATG GCACCCAGGT
G.elegans       CCTACGGCTT ATATTAAAAC CTTCCAAGGC CCACCGCATG GCATCCAGGT
G.hirsutum      CCTACGGCTT ATATTAAAAC CTTCCAAGGC CCACCGCATG GCATCCAGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       460        470        480        490        500
G.sylvestr      TGAGAGAGAT AAATTGAACA AATATGGTCG TCCCCTGTTG GGATGTACTA
G.latIfoli      TGAGAGAGAT AAATTGAACA AATATGGTCG TCCCCTGTTG GGATGTACTA
G.elegans       TGAGAGAGAT AAATTGAACA AATATGGTCG TCCCCTGTTG GGATGTACTA
G.hirsutum      TGAGAGAGAT AAATTGAACA AATATGGTCG TCCCCTGTTG GGATGTACTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       510        520        530        540        550
G.sylvestr      TTAAACCAAA ATTGGGGTTA TCAGCTAAAA ACTACGGTAG GGCGGTTTAT
G.latIfoli      TTAAACCAAA ATTGGGGTTA TCAGCTAAAA ACTACGGTAG GGCGGTTTAT
G.elegans       TTAAACCAAA ATTGGGGTTA TCAGCTAAAA ACTACGGTAG GGCGGTTTAT
G.hirsutum      TTAAACCAAA ATTGGGGTTA TCAGCTAAAA ACTACGGTAG GGCGGTTTAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       560        570        580        590        600
G.sylvestr      GAATGTCTTC GTGGTGGACT TGATTTTACC AAAGATGATG AAAACGTGAA
G.latIfoli      GAATGTCTTC GTGGTGGACT TGATTTTACC AAAGATGATG AAAACGTGAA
G.elegans       GAATGTCTTC GTGGTGGACT TGATTTTACC AAAGATGATG AAAACGTGAA
G.hirsutum      GAATGTCTTC GTGGTGGACT TGATTTTACC AAAGATGATG AAAACGTGAA
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                       610        620        630        640        650
G.sylvestr      CTCCCAACCG TTTATGCGTT GGAGAGATCG TTTCTTGTTT TGTGCCGAAG
G.latIfoli      CTCCCACCCG TTTATGCGTT GGAGAGATCG TTTCTTGTTT TGTGCCGAAG
G.elegans       CTCCCAACCG TTTATGCGTT GGAGAGATCG TTTCTTGTTT TGTGCCGAAG
G.hirsutum      CTCCCAACCG TTTATGCGTT GGAGAGATCG TTTCTTGTTT TGTGCCGAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       660        670        680        690        700
G.sylvestr      CAATTTTTAA ATCACAGGCT GAAACTGGCG ---------- ----------
G.latIfoli      CACTT----- ---------- ---------- ---------- ----------
G.elegans       CAA------- ---------- ---------- ---------- ----------
G.hirsutum      CAATTTTTAA ATCACAGGCT GAAACTGGCG AAATCAAAGG ACATTACTTG

                ....|...
G.sylvestr      --------
G.latIfoli      --------
G.elegans       --------
G.hirsutum      AATGCTAC
```

There were a total of 644 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [Tamura *et al.,* 2013].

Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	*8.26*	*6.09*	11.89
T/U	*7.95*	-	6.78	*6.70*
C	*7.95*	9.20	-	*6.70*
G	14.11	*8.26*	*6.09*	-

NOTE.-- Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model [1]. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 100, The nucleotide frequencies are A = 27.41%, T/U = 28.49%, C = 21.00%, and G = 23.10%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -1033.849. The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 644 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2].

Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution

	A	T	C	G
A	-	*7.94*	*5.85*	13.17
T	*7.64*	-	6.57	*6.44*
C	*7.64*	8.91	-	*6.44*
G	15.63	*7.94*	*5.85*	-

Note:- Each entry shows the probability of substitution (r) from one base (row) to another base (column) [1]. For simplicity, the sum of r values is made equal to 100.

Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. The nucleotide frequencies are 27.41% (A), 28.49% (T/U), 21.00% (C), and 23.10% (G). The analysis involved 4 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 644 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [2].

DISCUSSION

Similar to the above there were molecular diversity in Gymnema sylvestre growing in different ecotypes, Reddi and Gopi 2008, did Molecular characterization/genetic diversity of the five different ecotypes of Gymnema sylvester was studied using Random Amplified Polymorphic DNA (RAPD) analysis. A preliminary study was carried out using only four primers namely, OPL 01, OPL 02, OPL 06, and OPL 07. Except OPL-01 Primer all the primers gave satisfactory amplification of 63 bands. OPL-01 Primer gave the monomorphic banding pattern. Out ofthe total 63 bands amplified, 18 (28.57%) were observed to be polymorphic and 5 (7.93%) were unique, i.e specific to a particular ecotype. Nair and R. Keshavachandran 2006, In the cluster analysis based on isozyme analysis also, 'Panniyur' remained separate showing a high level of variation. The alternate cluster of 16 accessions further segregated into three subclusters each with a number of sub-sub-clusters. The highest genetic similarity was noticed between 'Peringottukurishi 137' and 'Kuzhalmannom 89' with a similarity coefficient of 0.85. These two accessions had identical isozyme banding patterns also. The genetic divergence of 0.15 to 0.28 across the *Gymnema* accessions reflect considerable variations at the DNA level and indicates a wide and diverse genetic base of the materials studied. The sources of polymorphism also could be deletion of a priming site, insertions that render priming sites too distant to support amplification or insertions that change the size of a DNA segment without preventing amplification (Williams *et al*., 1990). Nonetheless, the collections originating from various

parts of the state did not form well-defined groups and were interspersed with each other, indicating little association between RAPD pattern and geographic origin of the accessions. This may be because of recurrent introduction from the same source or sharing of germplasm among the farming community across different geographical locations. On a final note, the present study revealed the existence of considerable variations at the molecular level in the *Gymnema* germplasm, which confirms the results of the earlier on morphological and biochemical studies. The results could be used for identification of ideal genotypes for extraction of drugs by correlating the molecular fingerprints with desirable morphological and biochemical features. It will also help in devising strategies to protect the genetic diversity of this species. And also the DNA barcoding reveals the genetic close proximity and genetic diversity at molecular levels gives the molecular evolutionary trends and internal compositional similarity and divergence among the Gymnema sps., can be further established.

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