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ORIGINAL ARTICLE

Molecular Identification and Next-Generation Sequence Analysis of Interspecies Genetic Variations among Three Varieties of *Datura*

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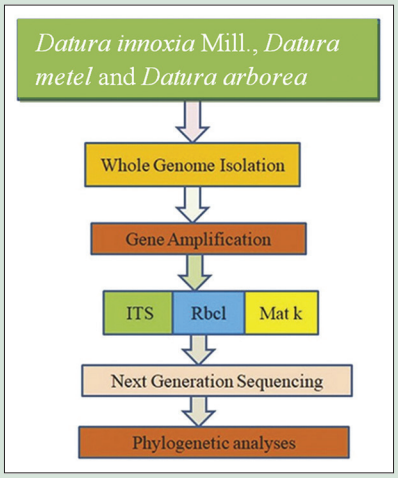
ABSTRACT

Background: *Datura* is a well-known plant used in Ayurveda. It is a widely growing plant from *Solanaceae* family, attributed to poisonous and medicinal values. It is used in the treatment of various skin diseases, fever, etc., It is also used for external application and rarely for oral administration. **Objectives:** The present study aims to compare three different varieties of *Datura* which include the species of *Datura innoxia* Mill., *Datura metel* L. and *Datura arborea* are used to study molecular marker and phylogenetic analysis. **Materials and Methods:** Whole-genomic DNA was isolated from the leaves of *Datura* and the polymerase chain reactions amplification of DNA barcoding markers are rbcl, mat k, and internal transcribed spacer-4 and 5 were analyzed by 0.8% agarose gel electrophoresis. **Results:** The DNA barcoding markers and next-generation sequencing are able to identify the interspecies genetic variations among these closely related plant varieties of *Datura*. **Conclusion:** The interspecies genetic variations among these closely related three species of *D. innoxia*, *metel*, and *arborea* was closely related with *Datura stramonium* isolate NN003 chloroplast genome similarity of 98%, 99%, and 99%, respectively.

Key words: Ayurveda, *Datura*, internal transcribed spacer 4, mat k, medicinal plants, next-generation sequencing, ribulose bisphosphate carboxylase/oxygenase form I gene

SUMMARY

- *Datura* is a widely growing medicinal plant belonging to the family of solanaceae
- The study is designed to evaluate the molecular identification of genetic variations among these closely related three varieties of *Datura* to understand the molecular similarities
- We have performed molecular markers such as rbcl, mat k, internal transcribed spacer, and next-generation sequencing
- The interspecies genetic variations among these closely related three species of *Datura innoxia*, *Datura metel*, and *Datura arborea* were closely related with *Datura stramonium*.



Abbreviations Used: Rbcl: Ribulose bisphosphate carboxylase/oxygenase form I gene; mat k: Maturase K; ITS: Internal transcribed spacer; PCR: Polymerase chain reactions; TAE: Tris-Acetate-EDTA buffer; BLAST: Basic Local Alignment Tool; MEGA: Molecular Evolutionary Genetics Analysis; rRNA: Ribosomal RNA.

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DOI: 10.4103/pr.pr_101_19

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INTRODUCTION

Datura is mentioned in ancient classical literature like Charaka by the name of Kanaka.[1] Sushruta described *Datura* for the treatment of dog bites. Bhavamishra described *Datura* leaf externally for the treatment of lice, for analgesic and anti-inflammatory actions.[2] Vangasena described *Datura* seeds are used after purification processes by boiling in cow milk and shade dried in the room temperature and used it for filariasis.[3] *Datura* is known by the name thorn apple is a poisonous herb. It is used in many Ayurvedic medicines after the purification method.[4] Leaves of *Datura* are used in alopecia, pain in the breast, tumor, rheumatism, and in inflammation and its flowers are anti-asthmatic. The leaves, seeds, and roots are anti-catarrhal and used in bronchial asthma, skin diseases, wounds, cough, burning micturition, mental illness, febrifuge, diarrhea, and dermatosis. Its chemical composition scopolamine, daturadiol, hyoscyamine, fastudine, allantoin, niacin, Vitamin C, atropine, noratropine, meteolodine, hyoscine, fastusic acid, etc.[5]

Among all its varieties, Krishna *Datura* (black) is attributed to prime potentialities. In Rajanighantu, five varieties of *Datura* have been

Cite this article as: Prasad GP, Pratap GP, Marimuthu S, Prasad SB, Rao G, Mangal AK, *et al*. Molecular identification and next-generation sequence analysis of interspecies genetic variations among three varieties of Datura. Phcog Res 2020;12:158-62.

mentioned based on the flowering color of the plant (white, blue, black, red, and yellow).[6] In this study, three varieties of *Datura* which include the species of *D. innoxia, D. Metel* and *Datura arborea* were studied. The aim of the present study is to evaluate the molecular identification of rbcl, mat k, and internal transcribed spacer (ITS) 4 and 5 universal genes and next-generation sequencing (NGS) are used to explore the interspecies genetic variation among these closely related three plant varieties.

MATERIALS AND METHODS

Chemicals

Plant DNA isolation kit was purchased from Favorogen, polymerase chain reactions (PCR) master mix (2X) from Genetix Biotech Asia Pvt. Ltd., New Delhi, India. Agarose, 6X gel loading dye and 100 bp and 1 Kb DNA ladder were procured from Himedia Laboratories PVT, LTD, Mumbai, India, Primers mat k, rbcl, and ITS-4 and 5 was synthesized by Eurofins Genomics India Pvt. Ltd, Bangalore, Karnataka, India. All other chemicals and solvents were of analytical grade and obtained from Himedia, Mumbai, India.

Plant collection

Fresh leaves of *Datura* are *D. Innoxia, D. metel* and *D. arborea* were collected from the Medicinal plant garden at Regional Ayurveda Institute for Fundamental Research (RAIFR), Kothrud, Pune, Maharashtra, India [Figure 1]. The plant specimen was authenticated by Dr. Gajendra Rao, Research officer an expert in the field of Botany and live plants are being maintained in the Arborium/Garden of in RAIFR, Pune, Maharashtra, India, with a registration number of DMA-01, DMI-02, and DMD-03.

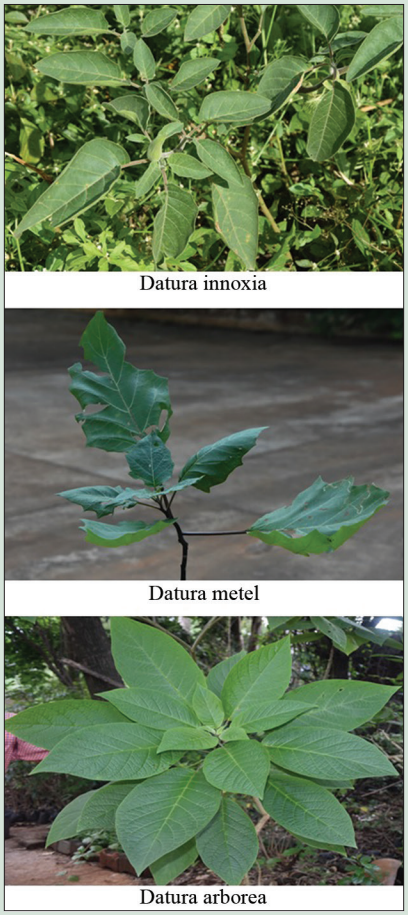

Figure 1: Photography images of three varieties of *Datura* species

Extraction of whole-genomic DNA

The leaves of *Datura* varieties *D. Innoxia Mill., D. metel* and *D. arborea* 100 mg of each leaf were crushed with liquid nitrogen into a fine powder. Total genomic DNA was extracted from all the samples using the Favorgen Plant genomic DNA extraction kit. A Nanodrop was used to determine the concentration of genomic DNA. The integrity of DNA was confirmed by visualization on 0.8% agarose gel using ethidium bromide staining.

Polymerase chain reactions amplification

The extracted DNA was used as a DNA template in the PCR reaction. PCR amplification was conducted in 25 µl of PCR mixture containing 12.5 µl of PCR master mix (2X), 10 µM forward and reverse primer (2 µl each), 3.5 µl nuclease-free water, and 10 ng of template DNA. The thermal cycling conditions were initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30-s annealing at 50°C for 1 min, extension at 72°C for 2 min followed by a final extension at 72°C for 10 min. The amplified products were detected by 0.8% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer and visualized with ethidium bromide staining. Primers used for amplification of ITS-5 and 4, mat k, and rbcl genomic regions shown in Table 1.

Amplification and next-generation sequence analysis

PCR was performed using the rbcl gene region. The reaction mixture was done in 25 µL consisting of the 12.5 µl of PCR master mix (2X), 0.25 µM forward and reverse primer, and 3.5 µl nuclease-free water and 10 ng of template DNA. The reaction mixture was incubated at 94°C for 1 min and amplification was performed with the following 35 thermal cycles; denaturation for the 30s at 94°C, annealing for 40s at 53°C, an extension for 40s at 72°C, and the final extension for 5 min at 72°C. The amplified PCR products were detected by 0.8% agarose gel electrophoresis in TAE buffer and visualized with ethidium bromide staining. Sequenced products were precipitated using two volumes of 80% propanol and then washed twice with 80% ethanol. The PCR-products were air-dried and resuspended in a denaturing buffer containing 15 µL of formamide. The final sequencing was done using Applied Biosystems DNA sequencer following standard protocol. Sequence analyzing was outsourced to Eurofins Genomics India Pvt. Ltd, Bangalore, Karnataka, India and was analyzed by NGS. The nucleotide sequence for rbcl gene sequence was subjected to sequence alignment using the Basic Local Alignment Tool (BLAST). The number of hits with homologous sequences is inferred based on similarity and alignment.

Phylogenetic tree

Molecular Evolutionary Genetics Analysis version 10, was used to construct a maximum likely hood tree for the obtained sequences to identify its interspecies relationships.

Table 1: Primers used for amplification of ITS-5 and 4, mat k, and rbcl genomic regions

Primer name	Sequences (5′-3′)
ITS5-forward	5’-GGAAGTAAAAGTCGTAACAAGG-3’
ITS4-reverse	5’-TCCTCCGCTTATTGATATGC-3’
mat k-forward	5’-TAATTTACGATCAATTCATTC-3’
mat k-reverse	5’-CTTCCTCTGTAAAGAATTC-3’
rbcl-forward	5’-ATGTCACCACAAACAGAGACTAAAGC-3’
rbcl-reverse	5’-GTAAAATCAAGTCCACCRCG-3’

ITS: Internal transcribed spacer; mat k: Maturase K; rbcl: Ribulose bisphosphate carboxylase/oxygenase form I gene

RESULTS

Genomic DNA quantification

The extracted plant DNA was analyzed using Nanodrop Eppendorf. The obtained DNA concentration from the *D. innoxia* was 20 ng/µl, *D. metel* was 25 ng/µl and *D. arborea* was 22 ng/µl, respectively. Hence, due to the good quality of genomic DNA concentrations in all three varieties of *Datura* was used as a DNA template for rbcl, mat k, and ITS-4 and 5 for further molecular marker analyses.

Internal transcribed spacer 4 and 5 gene amplification

ITS is the spacer DNA situated between the small subunit ribosomal RNA (rRNA) and large subunit rRNA genes in the chromosomes. Figure 2 shows the changes in the ITS-4 and 5 universal gene amplification to generate the PCR product approximately 670 base pairs (bp) in size of agarose gel electrophoresis. The gene was amplified successfully in *D. innoxia, D. metel* and *D. arborea*.

Rbcl and Mat k gene amplification

Figures 3 and 4 show the changes in the rbcl and mat k gene amplification to generate the PCR product of agarose gel electrophoresis. The results of DNA barcode performances with mat k and rbcl regions in the three varieties of *Datura*. The amplification of the gene was successful only in the rbcl gene region, The fragment sizes are about 550 bp. Mat k gene expression was less amplified when compared with rbcl gene. All the varieties of *Datura,* rbcl gene amplification are similar when compared with mat k gene.

Next-generation sequence analysis

Table 2 shows the statistical simulation of BLAST sequence homology of *D. innoxia, D. metel* and *D. arborea* species with rbcl primers, respectively. Sequence homology of the amplified sequence was detected using BLAST. The sequence length of rbcl was 614, 611 and 610 nucleotides with *D. innoxia*, *metel* and *arborea* varieties, respectively. Similarily with rbcl sequence homology of all the three species was 98%, 99%, and 98%, respectively.

Table 3 shows the identification of single gene mutation of three varieties of *D. innoxia*, *metel* and *arborea* varieties with rbcl primers, respectively. We observed that species-specific substitution as marker nucleotides because they may be crucial for identifying each species. In *D. innoxia,* there is a mismatch sequence which was observed at the position of 125, 129, 444, 459, 592, and 605 and insertion was observed at the position of 130–132 and 30134. In *D. metel* species the mismatch sequences were observed at the position of 437 and 607 nucleotide sequences and the insertion was observed at the position of 111, 25481, 58541–58542. In *D. arborea* species we found that mismatch sequences were observed at the position of 10, 12, 46, 437, 605, and 606, respectively, and the insertion was observed at the sequence of 141, 597–599.

Phylogenetic analysis

To evaluate the phylogenetic relationships among the three varieties of *D. innoxia*, *metel* and *arborea*, phylogenetic trees were constructed by applying the NJ method to the rbcl sequences. As shown in the result of the phylogenetic tree [Figure 5], when all the three DNA barcodes sequences were employed, *Datura metel* and *D. innoxia* were closer genetically similar when compared to *D. arborea*. Therefore, the rbcl barcode sequences provided higher resolution for the identification of the clusters that constitute clades within the same species. From these phylogenetic analyses, we confirm that the identification of the three varieties can be achieved using rbcl universal gene.

DISCUSSION

DNA barcoding is a popular method for species identification of fresh tender leaves. It is mainly used to verify plants that lack flowers or have incomplete morphological characteristics. It is also to be used in samples such as processed food, fossil remains and herbarium specimens, in which the DNA has been highly degraded.[7] It involves sequencing a standard region of DNA as a tool for species identification.[8] Rbcl, mat k, and ITS-4 and 5 possess attributes that are highly desirable in a plant DNA barcoding system, although none of the three loci fits all three criteria perfectly. Chase, in 2007, reported that using two barcoding region rbcl and mat k options as a standard protocol for barcoding of all land plants.[9] Consortium for the barcode of life's plant working group reported that DNA barcodes with mat k and rbcl gene loci are the use of two regions of plastid DNA as a standard protocol for the core barcoding of land plants.[10] In this study, we assessed the molecular markers of ITS-4 and 5, mat K, and rbcL and next-generation sequences are used to identify the interspecies genetic variations among these closely related three species of *Datura*.

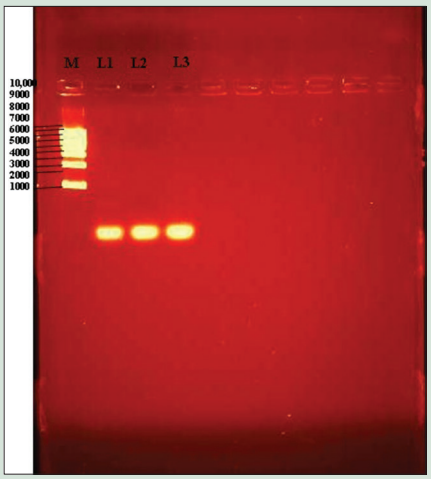

Figure 2: M represents marker 1 kb DNA ladder and Lanes L1 - *D. innoxia* of internal transcribed spacer, L2 - *D. metel* of internal transcribed spacer and L3 - *D. arborea* of internal transcribed spacer

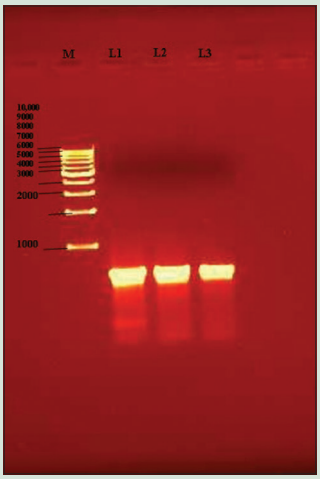

Figure 3: M represents marker 1 kb DNA ladder and Lanes L1 - *D. innoxia* of rbcl gene, L2 - *D. metel* of rbcl gene, L3 - *D. arborea* of rbcl gene

Table 2: Statistical simulation of Basic Local Alignment Tool sequence homology of *D. innoxia, D. metel* and *D. arborea* varieties with rbcl primers, respectively

Species	Scientific name	Reference ID	Best hit	Gene	Max score	Query cover (%)
Datura	*D. innoxia*	JN662489.1	*Datura stramonium*	RBCL	990	95
		MH311563.1	*Datura metel*	RBCL	966	95
Datura	*D. metel*	JN662489.1	*Datura stramonium*	RBCL	996	99
		KF365991.1	*Datura innoxia*	RBCL	981	97
Datura	*D. arborea*	JN662489.1	*Datura stramonium*	RBCL	990	98
		MH311563.1	*Datura metel*	RBCL	966	94

RBCL: Ribulose bisphosphate carboxylase/oxygenase form I gene

Table 3: Identification of single gene mutation of three varieties of *D. innoxia, D. metel,* and *D. arborea*

Species	Scientific name	Mismatch	Insertion
Datura	*D. innoxia*	125(G, T);129(C, G);444(A, G);459(A, G);592 (C, G);605(A, T)	130-132(TGA,---);30134(A,-)
Datura	*D. metel*	437(A, G); 607(A, C)	111(C,-);25481(C,-);58541-58542(AT,--)
Datura	*D. arborea*	10(A, C);12(C, A);46(G, A);437(A, G);605-606(TA, AC)	141(C,-);597-599(GAA,---)

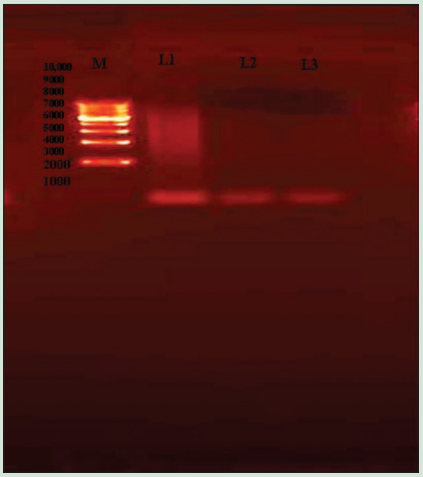

Figure 4: M represents marker 1 kb DNA ladder and Lanes L1 - *D. innoxia* of mat k gene, L2 - *D. metel* of mat k gene, L3 - *D. arborea* of mat k gene

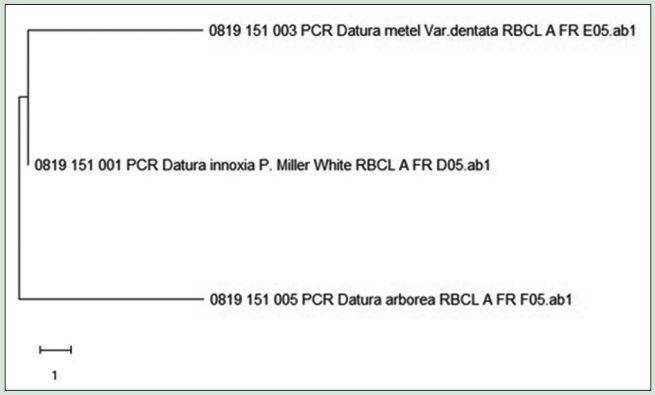

Figure 5: Phylogenetic tree showing common linkeage between *D. innoxia, D. metel* and *D. arborea*

The results of PCR amplification revealed that the two molecular markers ITS-4 and 5 and rbcl genes are amplified successfully, but in the case of mat k gene was not amplified when compared with the other two genes. The inter and intraspecific distances of ITS were greater than those of mat k and rbcl. Various studies have shown that the interspecific sequence divergence among DNA barcode regions is the highest in ITS followed by mat k and rbcl region. DNA barcode regions in plant systems can be divided into protein-coding and noncoding regions. Noncoding regions such as the nuclear ribosomal ITS, whereas protein-coding regions such as mat k and rbcl.[11]

NGS is another facility of advanced genomics era to have a more precise picture of species genome and to identify more orthologous and paralogous regions at different loci of different species.[12] The sequencing of the entire plastid genome along with ITS is becoming a method of choice for species discrimination. In the present study, we amplified rbcl gene region and sequenced the conserved regions in three varieties of *D. innoxia*, *metel* and *arborea*. Sequence homology of the amplified sequences was detected using BLAST.[13] In the present study, we have identified the rbcl gene sequence homology of all the three varieties of *D. innoxia*, *metel* and *arborea*. *D. arborea* was 98% related to *Datura stramonium* and 94% related to *Datura metel*. *D. innoxia* 95% related to *D. stramonium* and 95% related to *Datura metel*. *Datura metel* closely related to *D. stramonium* 99% and *D. innoxia* 97%, respectively.

CONCLUSION

Thus, from the results obtained, we observed that the molecular markers of ITS-4 and 5, mat k, and rbcl gene amplification and sequencing are used to identify the interspecies genetic variations among these closely related three varieties of *D. innoxia*, *D. metel,* and *D. arborea* was closely related with *D. stramonium* isolate NN003 chloroplast genome similarity of 98%, 99%, and 99%, respectively. *Datura metel* and *innoxia* were closer genetically similar when compared to *D. arborea* variety. Rbcl barcode sequences provided higher resolution for the identification of the clusters that constitute clades within the same species. Phylogenetic analyses confirm that the identification of the three varieties of *Datura metel* can be achieved using rbcl universal gene sequence analysis.

Acknowledgements

The authors express their sincere thanks to the Central Council for Research in Ayurvedic Sciences, New Delhi, India, for providing financial assistance and all necessary facilities.

Financial support and sponsorship

CCRAS, New Delhi, India.

Conflicts of interest

There are no conflicts of interest.

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