Pharmacogn. Mag.
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ORIGINAL ARTICLE

DNA Barcoding Markers to Identify Intraspecies Genetic Variations in Three Ecotypes of *Abrus precatorius* L.

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Submitted: 01-Feb-2020 Revised: 29-May-2020 Accepted: 06-Jul-2020 Published: 30-Nov-2020

ABSTRACT

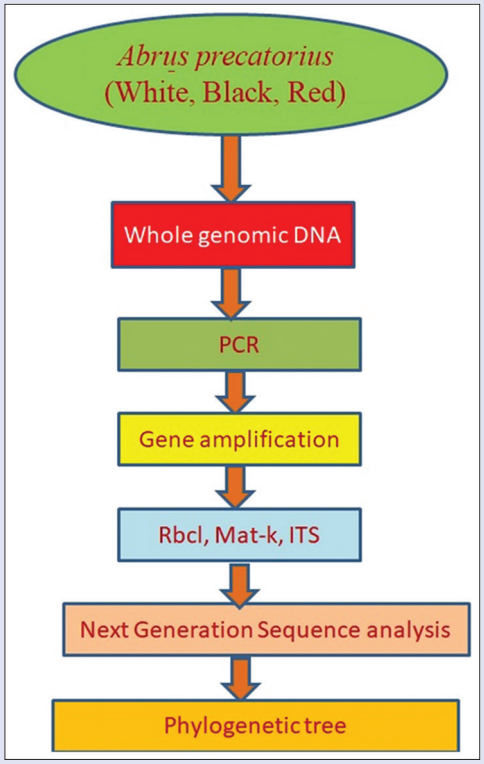
Background: *Abrus precatorius* is also called as Gunja in Ayurveda. It is a commonly grown plant belonging to the family of *Fabaceae*. It is characterized under the Upavisha (semi-poisonous drugs) and used widely in various Ayurvedic formulations with great beneficial significance. It is used in the treatment of various diseases such as alopecia, edema, helminthiasis, skin diseases, itching, and urinary disorders after being passed through specific purification procedures. **Objectives:** The present study aims to compare three different varieties of *A. precatorius* which includes the species of white, black, and red which are used to study DNA barcoding marker and phylogenetic analysis. **Materials and Methods:** Whole genomic isolated DNA from three varieties (white, black, and red) of *A. precatorius* leaves and subjected to analysis of the polymerase chain reaction of rbcL, maturase K, and internal transcribed spacer 4,5 using 0.8% agarose gel electrophoresis. **Results:** The DNA barcoding markers and next-generation sequencing can identify the intraspecies genetic variations among these closely related plant varieties of *A. precatorius* of white, black, and red. **Conclusion:** The intraspecies genetic variations among these three varieties of *A. precatorius* white, black, and red are closely related with *A. precatorius* isolate TMP 144 Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit gene 99.81%.

Key words: *Abrus precatorius*, ayurveda, internal transcribed spacer 4, maturase K, medicinal plants, next-generation sequencing, Rbcl

SUMMARY

- *Abrus precatorius* is also called as Gunja in Ayurveda
- The study is designed to evaluate the molecular identification of genetic variations among these closely related three varieties of *Abrus precatorius* to understand the molecular similarities
- We have performed molecular markers such as rbcL, maturase K, internal transcribed spacer, and next-generation sequencing
- The intraspecies genetic variations among these closely three varieties of *A. precatorius* white, black, and red are closely related with *A. precatorius* isolate TMP 144 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene of 99.81%.

Abbreviations used: rbcL: Ribulose bisphosphate carboxylase/oxygenase form I gene; matK: 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/pm.pm_23_20

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INTRODUCTION

Abrus precatorius L. is a glabrous wiry climber. It is grown in tropical and subtropical regions.[1] Gunja is the Visha Dravya mentioned in the ancient classical literature like Samhita and other Ayurvedic texts.[2] In Samhita, it is mentioned under Sthavara Visha and in the texts of Rasashastra, it is classified under Upavishas.[3] Aacharya Charaka mentioned this drug in Vajikarana Adhyaya and Aacharya Sushruta classified Gunja under MoolaVisha (root poison).[4] In

Cite this article as: Marimuthu S, Pratap GP, Prasad GP, Prasad SB, Mangal AK, Srikanth N. DNA barcoding markers to identify intraspecies genetic variations in three ecotypes of *Abrus Precatorius* L.. Phcog Mag 2020;16:S540-5.

Bhavaprakasha Nighantu, it is mentioned under Guduchyadi Varga (group starting with Guduchi-*Tinospora sinensis* (Lour.) Merr.), Yogaratnakara and other Rasashashtra texts mentioned it under Upavisha (semi poisonous group).[5] The two characteristics of Gunja are mentioned, i.e., Shweta Gunja and Rakta Gunja. Out of this, Shweta Gunja (white variety) is considered as highly toxic.[6] Several synonyms are mentioned in Ayurvedic and other existing texts. The Rakta Gunja synonyms are Gunja, Chudamani, Tamrika, Raktika, Rakta, and Shitapaki, whereas the synonyms of Shweta Gunja are Shweta kambhoji, Kakapilu, Kakadani, Bhirintika, and Vaktrashalya.[7] The common names of Gunja are rosary pea, Indian bead, and Buddhist rosary bead. There is a difference of opinion among the Aacharya's concerning the identification of the plant Gunja based on synonyms. Some controversial synonyms are Uchchata, Kakadani, Chudamani, and Shweta kambhoji.[8] Although in classical literature, black variety is not mentioned. In Andhra Pradesh forest regions, a black variety of Gunja is also available. The vegetative parts and total habit of these three varieties appear similar, but flowering and stage-wise fruiting

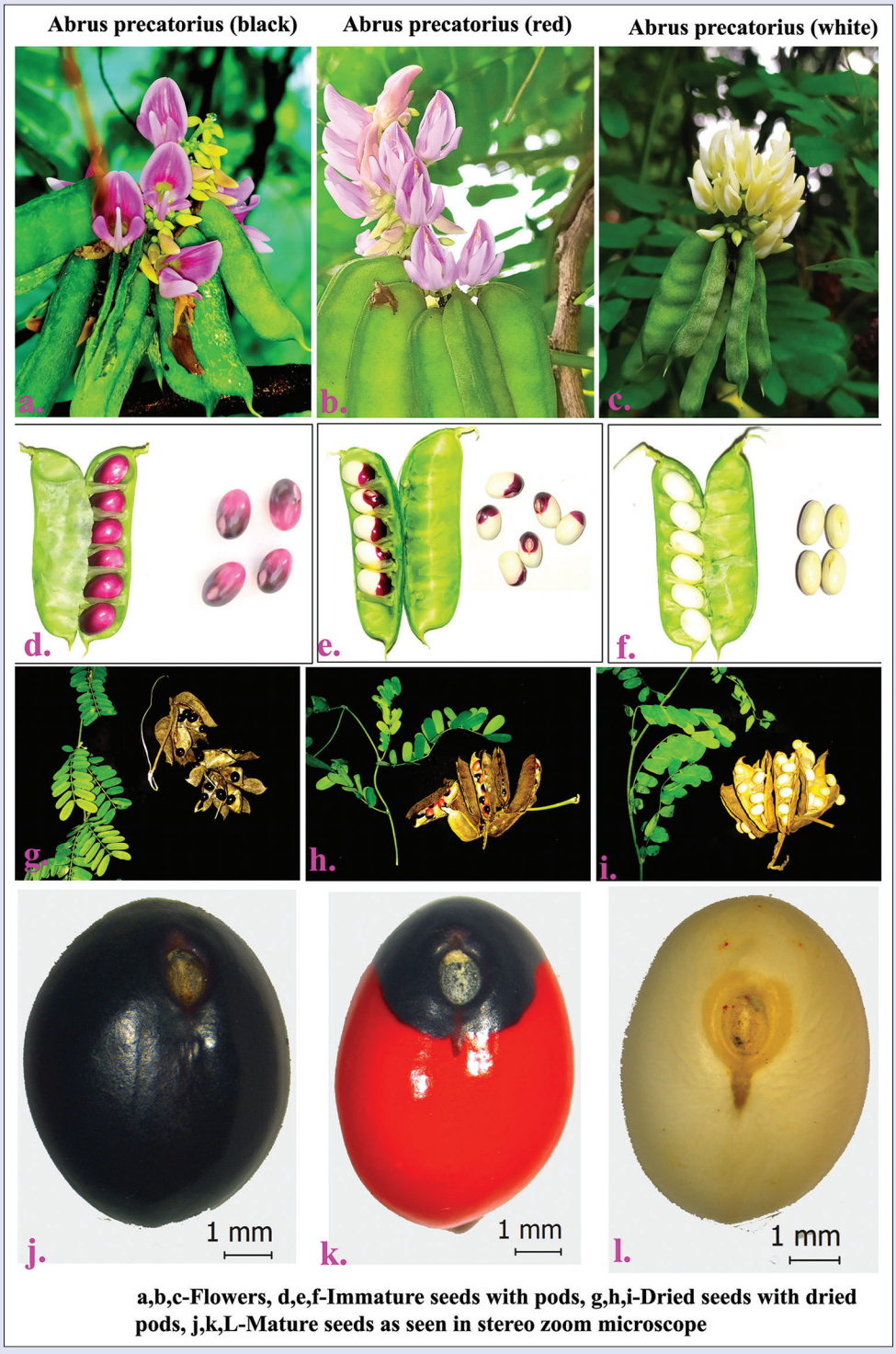

Figure 1: Comparative morphological characters of *Abrus precatorius*

gradually change their color, as shown in Figure 1. Red-seeded variety shows light pink color flowering, white-seeded variety shows flowering in creamy white color, and black-seeded variety shows a dark pink color. The seeds in immature pods of red-seeded variety appear in pink and white color, and in the mature seeds, this pink color gradually changes into black and white color turns into red color. In the white variety, the immature and mature pods appear in white only. In the black-seeded variety, the immature pods have only pink color and this pink color gradually changed into complete black color in mature pods. These findings provoked us to conduct the present study to find out genetic modifications at the DNA level to make it clear they are intraspecific changes for the formation of ecotypes. In this study, we comparatively analyzed the three DNA barcode regions, internal transcribed spacer (ITS), maturase K (matK), and rbcL, and next-generation sequencing is used to evaluate their ability to identify the intraspecies genetic variation among these closely related three varieties of *A. precatorius*.

MATERIALS AND METHODS

Chemicals

Plant DNA isolation kit was purchased from Favorogen, Polymerase chain reactions (PCR) Master Mix (×2) from Genetix Biotech Asia Pvt. Ltd., New Delhi, India. Agarose, 6X gel loading dye, 100 bp, and 1 kb DNA ladder were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Primers such as matK, rbcL, and ITS-4 and 5 were synthesized by Eurofins Genomics India Pvt. Ltd., Bangalore, Karnataka, India.

Plant collection

Fresh leaves of *A. precatorius* of white, black, and red were collected from Talakona forest, Mamandur, Chittor district, Andhra Pradesh, and nearby forest areas from Pune, Maharashtra state. These three varieties are identified at Regional Ayurveda Institute for Fundamental Research (RAIFR), Kothrud, Pune, Maharashtra, India. Live plants are being maintained in the herbarium/garden of RAIFR, Pune, Maharashtra, India, with a herbarium accession number of white, black, and red being SVUBS1215, SVUBS1672, and SVUBS738.

Extraction of whole genomic DNA

The leaves of *A. precatorius* varieties of white, black and red, 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 Favorogen 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 (×2), 10 µM forward and reverse primer (2 µl each), 3.5 µl nuclease-free wate, r 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-sec annealing at 50°C for 1 min, and 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, matK, and rbcL genomic regions are shown in Table 1.

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

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

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 (×2), 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 2 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 next-generation sequencing. The nucleotide sequence for rbcL gene sequence was subjected to sequence alignment using the Basic Local Alignment Search Tool (BLAST). The number of hits with homologous sequences is inferred based on similarity and alignment.

Phylogenetic tree

Molecular Evolutionary Genetics Analysis version 10 (The Pennsylvania State University, University Park, PA, USA) was used to construct a maximum likely hood tree for the obtained sequences to identify its intraspecies relationships.

RESULTS

Genomic DNA quantification

The extracted plant DNA was analyzed using Nanodrop Eppendorf. The obtained DNA concentration from the *A. precatorius* of white was 20 ng/µl, *A. precatorius* of black was 23 ng/µl, and *A. precatorius* of red was 25 ng/µl. Good quality of genomic DNA concentration were obtained in three varieties of *A. precatorius* which were subjected to amplification of DNA templates for rbcl, matk, ITS-4 and 5 for further molecular marker analysis.

Internal transcribed spacer and gene amplification

ITS of nuclear ribosomal DNA is a small nonfunctional RNA situated between structural ribosomal RNAs (rRNA) of a common precursor transcript, which is mainly useful for elucidating relationships among closely related generic species. Figure 2 shows the changes in the ITS-4 and 5 universal gene amplification to generate the PCR product approximately 670 base pairs (bps) in size of agarose gel electrophoresis. The gene was amplified successfully in *A. precatorius* of white, black, and red varieties.

Rbcl and maturase K gene amplification

Figure 3 shows the changes in the rbcL and matK gene amplification to generate the PCR product of agarose gel electrophoresis. The results of DNA barcode markers of mat k and rbcl gene regions was amplified in the three varieties of *A. precatorius*. The amplification of the gene was successful in rbcL gene region, the fragment sizes are about 550 bp. Mat k gene expression was amplified successfully in all varieties of white, black, and red *A. precatorius* when compared with rbcL gene, the bp size of the amplified gene was around 889 bp. All three varieties of *A. precatorius* rbcL gene amplification are similar when compared with matK gene.

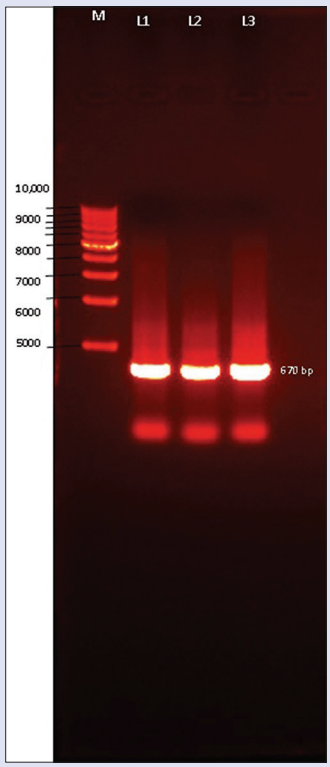

Figure 2: M represents marker 1 kb DNA ladder and Lanes L1 – *Abrus precatorius white* of Internal transcribed spacer, L2 – *Abrus precatorius black* of Internal transcribed spacer, and L3 – *Abrus precatorius red* of Internal transcribed spacer

Next-generation sequence analysis

Table 2 shows statistical simulation of BLAST sequence homology of *A. precatorius* of white, black, and red varieties with rbcL primers. Sequence homology of the amplified sequence was detected using BLAST. The sequence length of *A. precatorius* of rbcL was 614, 610, and 610 nucleotides match with *A. precatorius* of white, black, and red varieties, respectively. The interspecies genetic similarity among three varieties of *A. precatorius* are 99.81%.

Table 3 shows identification of single-gene mutation of three varieties of *A. precatorius* of white, black, and red varieties with rbcL primers. We observed species-specific substitution as marker nucleotides because they may be crucial for identifying each species. In *A. precatorius* of white, there is a mismatch sequence that was observed at the position of 24(G-A), 586(C, G), 590(T, G), and 593(A, G) and deletion was observed at the position of 415 (---,A). In *A. precatorius* of the black variety, the mismatch sequences were observed at the position of

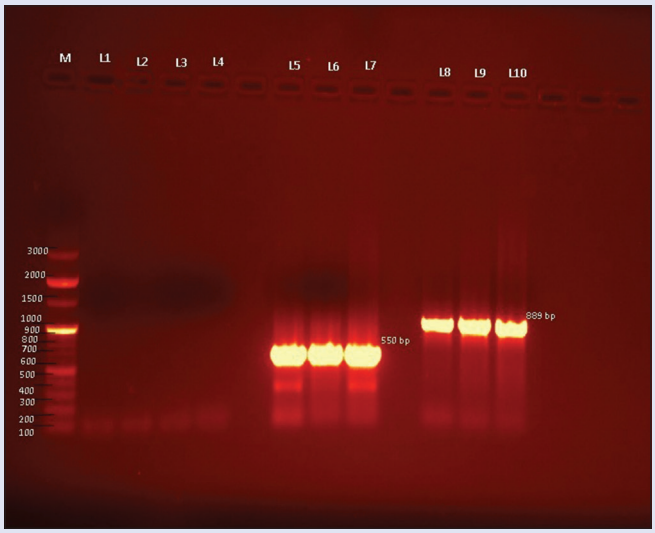

Figure 3: M represents marker 100 bp DNA ladder and Lanes L1 – Forward primer control of rbcL gene, L2 – Reverse primer control of rbcL gene, L3 – Forward primer control of Maturase K gene, L4 – Forward reverse control of Maturase K gene, L5 – *Abrus precatorius white* of rbcL gene, L6 – *Abrus precatorius black* of rbcL gene, L7 – *Abrus precatorius red* of rbcL gene, L8 – *Abrus precatorius white* of Maturase K gene, L9 – *Abrus precatorius black* of Maturase K gene, L10 – *Abrus precatorius red* of Maturase K gene

Table 2: Statistical simulation of basic local alignment tool sequence homology of *Abrus precatorius* of white, black, and red varieties with rbcL primers

Species	Scientific name	Reference ID	Best hit	Gene	Length	Percentage identification
White	*Abrus precatorius*	KF432060.1	*Abrus precatorius* isolate TMP 144 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene	rbcl	614	99.81
Black	*Abrus precatorius*	GU135185.1	*Abrus precatorius* voucher J.R. Abbott 24803 (FLAS) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene	rbcl	610	99.81
Red	*Abrus precatorius*	JN407281.1	*Abrus precatorius* isolate shawpc0583L ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene	rbcl	610	99.81

Table 3: Identification of single-gene mutation of three varieties of *Abrus precatorius* of white, black, and red

Species	Scientific name	Mismatch	Deletion
White	*A. precatorius*	24(G, A);586(C, G);590(T, G);593(A, G)	415 (-, A)
Black	*A. precatorius*	13(C, A);15(C, A);22(G, A);589(T, G);592(A, G)	-
Red	*A. precatorius*	22(G, A);589(T, G);592(A, G)	-

A. precatorius: Abrus precatorius

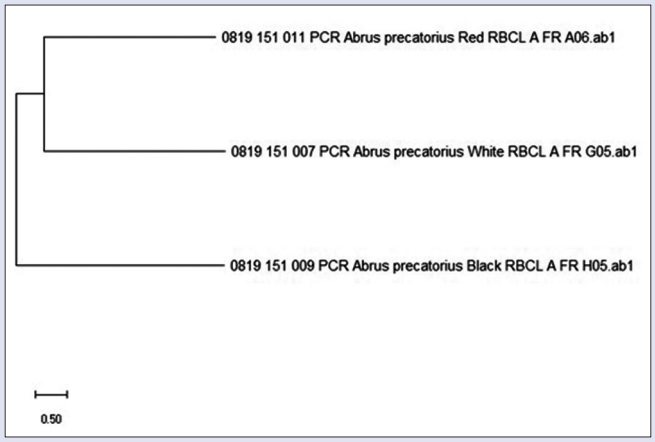

Figure 4: Phylogenetic tree showing the common linkage between *Abrus precatorius* white, black, and red

13(C, A), 15(C, A), 22(G, A), 589(T, G), and 592(A, G) nucleotide sequence and the deletion was not observed. In *A. precatorius* of the red variety, we found that mismatch sequences were observed at the position of 22(G, A), 589(T, G), and 592(A, G) and the deletion was not observed.

Phylogenetic analysis

To evaluate the phylogenetic relationships among the three varieties of *A. precatorius* of white, black, and red, phylogenetic trees were constructed by applying the Neighbor-Joining method to the rbcL sequences. As shown in the result of the phylogenetic tree [Figure 4] when all the three DNA barcodes sequences were employed, *A. precatorius* of red and *A. precatorius* of white were closer genetically and similar when compared to *A. precatorius* of black. 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 the rbcL universal gene.

DISCUSSION

Traditional herbs have been used in Ayurvedic medical systems since ancient times. According to the World Health Organization guidelines, authenticity, purity, and safety are important aspects of standardization and evaluation of traditional medicines.[9] DNA barcoding has many applications in various fields like phylogenetic analysis, authentication, interspecies and intra-species diversity and identification of medicinal plants etc.[10] The universal barcode loci for plants such as rbcL, matK, trnH-psbA, ITS, and 18S rRNA candidate regions are being used as DNA barcodes in plants.[11] In this present study, we assessed the DNA barcoding markers of rbcL, ITS-4 and 5, and Mat-k, and next-generation sequences are used to identify the intragenetic variations among these closely related three varieties of *A. precatorius*. The plastid DNA sequences have been used for the focus of DNA barcodes for plants.[12] The Plant Working Group of the Consortium for the Barcode of Life recommends using a combination of rbcL and matK regions as a universal DNA barcode Plantae.[13] The results of PCR amplification revealed that the three molecular markers of ITS, rbcL, and Mat k genes are amplified successfully and there is no difference among three varieties of *A. precatorius*. These DNA barcode regions have been used to identify the intraspecies genetic variation among these closely related three varieties of *A. precatorius,* the intraspecies distance of rDNA-ITS was greater than those of matK and rbcL.[14] Our results have shown that intraspecific distances are closely related to ITS locus followed by matK and rbcL genes in the three varieties of *A. precatorius*. In this study, we amplified rbcL gene region and sequenced the conserved regions in three varieties of *A. precatorius* white, black, and red varieties. Sequence homology of the amplified sequences was detected using BLAST.[15] In this study, we have identified the rbcL gene sequence homology of all the three varieties of *A. precatorius* white, black, and red varieties. *A. precatorius* white was 99.81% homology related to *A. precatorius* isolate TMP 144 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene. *A. precatorius* black was 99.81% homology related to *A. precatorius* voucher J. R. Abbott 24803 (FLAS) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene. *A. precatorius* red was 99.81% homology related to *A. precatorius* isolate shawpc0583 L ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene.

CONCLUSION

Thus, from the results obtained we observed that the DNA barcoding markers of ITS, matK, and rbcL gene amplification and sequencing are used to identify the intraspecies genetic variations among these closely related three varieties of *A. precatorius* white, black, and red. These three were closely related with *A. precatorius* isolate TMP 144 ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene (99.81%). Phylogenetic analyses confirm that the identification of the three varieties of *A. precatorius* is three ecotypes with intraspecies genetic variations.

Financial support and sponsorship

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

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Brijesh K, Kamath M. A review on toxic Drug-Gunja. J Ayurveda Integrated Med Sci 2017;2:181-3.
2. Sud S, Sud KS. Efficacy and usefulness of Visha Dravyas in Rasaushadhis (HMPs). J Tradit Med Clin Natur 2018;7:2.
3. Shastri K, Rasatarangini S.S. Rasatarangini. Ch.6. New Delhi; Motilal Banarasidas; 2002. p. 163-7.
4. Acharya R, Roy S. A review on therapeutic utilities and purificatory procedure of Gunja (*Abrus precatorius* L.) as described in Ayurveda. J Agric Sci Technol 2013;2:1-1.
5. Chunekar SK, Pandey DG, Nighantu BP. Guduchyadi Varga. Bhavaprakash Nighantu. Varanasi: Chaukhamba Bharati Academy; 2006. p. 269.
6. Shastri VL, Brahmashankar Sastri. Yogaratnakara. Chaukhambha Surbharati Prakashan, Varanasi, India. 2007. p. 167-8.
7. Sankhyadhar SC, Sri Narhari Pandit's. Raj Nighantu. Chaukambha Orientalia, Varanasi, India. 2012: p. 68-9.
8. Patel MH, Doshi KA, Winston C. Gunja (*Abrus precatorious* L.)–a noxious plant-a review. Pharma Science Monitor. 2015;6:41-55.
9. Hivale US, Bhatted SK, Bhojani MK, Bhusal N. A clinical study on the effect of Triphaladi Kala Basti with Arjuna Punarnavadi Ghanavati in the management of essential hypertension. Ayu 2018;39:250-5.
10. Feng S, Jiang M, Shi Y, Jiao K, Shen C, Lu J, *et al.* Application of the Ribosomal DNA ITS2 Region of Physalis (Solanaceae): DNA Barcoding and Phylogenetic Study. Front Plant Sci 2016;7:1047.

11. Bansal S, Thakur S, Mangal M, Mangal AK, Gupta RK. DNA barcoding for specific and sensitive detection of *Cuminum cyminum* adulteration in *Bunium persicum*. Phytomedicine 2018;50:178-83.
12. Kreuzer M, Howard C, Adhikari B, Pendry CA, Hawkins JA. Phylogenomic approaches to DNA barcoding of herbal medicines: Developing clade-specific diagnostic characters for Berberis. Front Plant Sci 2019;10:586.
13. Group CP, Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, *et al*. A DNA barcode for land plants. Proceed Natl Academy Sci 2009;106:12794-7.
14. Tahir A, Hussain F, Ahmed N, Ghorbani A, Jamil A. Assessing universality of DNA barcoding in geographically isolated selected desert medicinal species of Fabaceae and Poaceae. PeerJ 2018;6:e4499.
15. Wattoo JI, Saleem MZ, Shahzad MS, Arif A, Hameed A, Saleem MA. DNA Barcoding: Amplification and sequence analysis of rbcL and matK genome regions in three divergent plant species. Adv Life Sci 2016;4:03-7.