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DNA barcoding for species identification and phylogenetic investigation employing five genetic markers of *Withania coagulans*

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ABSTRACT

Withania coagulans is an important medicinal plant in India, dispersed from the East Mediterranean to South Asia, but *W. coagulans* is usually misidentified with other *Withania* species. Accurate identification of medicinally important plant species is helpful for their effective use in medicine and would help to protect the globally declining threatened or endangered plants. The present study aimed to create barcodes for *W. coagulans* using five genetic markers (*rbcl*, *matK*, *ITS*, *psbA-trnH*, and *rpoB-trnCGAR*) from two samples of *W. coagulans* collected at ICAR-Anand and the Institute of Science, Mumbai, to test the discrimination capacity of the plant DNA barcode. The study findings confirmed that *psbA* and *rbcl* are better barcodes for investigating *W. coagulans*, which displayed 100% conservation even when geographical sites were changed, whereas the genetic loci *rpoB, ITS,* and *matK* helped to distinguish between the distinct evolutions of the *Solanaceae* family. *ITS* showed the highest GC content, 66.9% for WCNB1 and 62.6% for WCNB2. The maximum likelihood *rpoB* marker gave the highest probability value (–889.38), followed by *rbcl* (–967.83) compared to other genetic markers. The study conclusions would be used in the pharmaceutical sector to develop DNA-based species identification of the *W. coagulans* plant to point out adulteration while plant collection. The work provides insights into molecular-based identification and authentication of *W. coagulans*.

1. INTRODUCTION

Withania coagulans, known as Rishyagandha, belong to the genus *Withania* of the *Solanaceae* family; due to *Withania's* rich pharmacological characteristics, it is one of India's most well-known genera of medicinal plants [1]. However, only two of the 23 species of *Withania* recognized, *Withania somnifera*, and *W. coagulans*, are of substantial economic importance [2]. *W. coagulans* is a grayish-white shrub common in East India, Nepal, and Afghanistan; in India, it occurs in Punjab, Rajasthan, Simla, Kumaun, and Garhwal [3]. *W. coagulans* have been suggested for treating several illnesses in traditional medical systems, including anti-tumor, anti-hyperglycemic, anti-diabetic, hepatoprotective, cardiovascular, immunosuppressive, hypolipidemic, free radical scavenging, and anti-depressant properties in the central nervous system [4–6]. In addition, *W. coagulans* has distinctive properties, making them a unique phytochemical.

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While the global acceptance of plant-based herbal therapy is increasing, the adoption of false substitution and adulteration is rising. The use of non-authentic herbal materials not only reduces the efficacy of herbal medicine but also creates safety issues. Despite its significant therapeutic value and widespread use, investigations on the authenticity of *W. coagulans* market samples are rare. Since *W. coagulans* and *W. somnifera* market samples show resemblance; in these circumstances, non-morphological DNA barcoding is quite useful for species identification and authentication [7].

DNA barcoding represents one of the most molecular biological marker-based techniques for determining target plant species in a short time. The goal of DNA barcoding, which is a widely used technology, is to identify various plant species utilizing nucleotide sequences accurately. Paul Hebert and colleagues were the first to propose using short DNA sequences known as DNA barcodes to identify biological organisms [8]. DNA barcoding studies can be used to authenticate plant species. The Plant Working Group, Consortium for the Barcode of Life (CBOL), recommends using the chloroplast genes *matK*, *rbcl*, and the intergenic spacer *trnH-psbA*, in addition to the nuclear region *ITS*, for DNA barcoding in plants [8–10]. All plant tissues contain DNA, a macromolecule more stable than RNA. Therefore, DNA-based markers are preferable for accurately identifying medicinal plants [11].

The present study aimed to identify differences in the gene sequences of two samples of *W. coagulans* grown in Gujarat and Mumbai by amplifying the *psbA-trnHf*, *rpoB*, *rbcl*, *matK*, and *ITS* gene markers from each sample, taking into account that environmental factors affect the physical characteristics of most plants [12]. Studies goal is to find the ideal barcode to identify and authenticate the plant. The sequences acquired will also be used to build a phylogenetic tree and to see its interrelationships with other *Solanaceae* plant species. It also aims to discover the best genetic marker for *W. coagulans* barcoding studies.

2. MATERIALS AND METHODS

2.1. Plant Sample Collection and Authentication

Plant sample 1 (WCNB1) of *W. coagulans* was obtained from the ICAR-Directorate of Medicinal Aromatic Plants Research in Anand, Gujarat, India (22°35'57.2"N 72°55'58.9" E). The seeds were collected from ICAR-Gujarat and were grown at The Institute of Science, Mumbai; it was referred to as plant sample 2 (WCNB2). The voucher herbarium specimen prepared was deposited to the Department of Botany, Shivaji University, Kolhapur, for authentication.

2.2. DNA Isolation

DNA isolation was carried out by taking 1 g of cleaned plant fresh leaf sample crushed in mortar and pestle using liquid nitrogen. 2% cetyltrimethylammonium bromide (CTAB), 100mMTris- pH 8.0, 20mM EDTA, and 1.7 M NaCl with 0.3% (v/v) β-mercaptoethanol were used as a lysis buffer for isolation of total genomic DNA. The pellet obtained was suspended in 30 µl TE buffer. The quality of the isolated DNA was analyzed on 0.8% agarose gel electrophoresis, and the quantity was checked using a NanoDrop spectrophotometer. The extracted DNA was stored chilled at –20°C [13].

2.3. PCR Amplification and Sequencing

Polymerase chain reaction (PCR) is used to amplify five genetic markers from *W. coagulans*: Two intergenic spacer regions (*trnH-psbA, rpoB-trnCGAR*), *ribulose 1, 5 bisphosphate* (*rbcl*), one nuclear region *Inter Transcribed Spacer* (ITS) in a 25 µl reaction mixture, and *maturase kinase* (matK) in a 50 µl reaction mixture. The reaction mixture of 25 µl contained 100 ng DNA templet, 0.5 U DreamTaq DNA Polymerase enzyme (Thermo Fischer), 10 mM dNTPs, 2.5 mM $MgCl_2$, 10X DreamTaq Buffer, and 0.5 µM forward and reverse primers (Eurofins, India). The list of forward and reverse primers, annealing temperature (Tm), and cycling conditions used in this study is mentioned [Table 1]. A thermal cycler (Applied BioSystems) was used for the PCR reaction. The amplified product was observed for quality using 1.5% agarose gel electrophoresis (1x TAE buffer and 0.5 µg/mL ethidium bromide). All samples were cleaned using the Qiagen Gel Extraction kit to remove extra primer dimers, templates, and dNTPs. The samples were then transported to Eurofins India Pvt. Ltd. for Sanger sequencing.

2.4. DNA Sequence Data Analysis

The nucleotide sequences from *W. coagulans* were compared to sequences found in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) for similarity match. The sequences obtained were then submitted to NCBI GenBank. Their accession numbers are cited [Table 2]. Published sequences were retrieved for additional analysis. Then, multiple sequence alignment was performed with retrieved sequences, and Finch TV was used to edit them. MEGA 11 (Molecular Evolutionary Genetic Analysis) software was used to evaluate parameters including genetic distance, parsimony informative sites, conserved sites, and variables sites [14]. Maximum likelihood and Tamura-Nei Model were used to build a phylogenetic tree with 1000 bootstrap replications. As out-groups, taxa from the *Ipomoea* and *Convolvulus* genes were utilized.

3. RESULTS

3.1. Results of PCR and DNA Barcode Sequence Analysis

In the present study, the ten sequences obtained from the two samples of *W. coagulans* grown in Mumbai and collected from ICAR-Anand

Table 1: List of primers and cycling conditions for DNA amplification.

DNA marker	Primer sequence	Tm in degree	Cycling conditions
psbA-trnHf			
Forward primer	5' GTTATGCATGAACGTAATGCTC 3' [20]	58° C	95°-5 min, 94° – 1 min, 58° – 1 min, } 35 Cycles
Reverse primer	5' CGCGCATGGTGGATTCACAATCC 3' [20]		
rpoB-trnCGAR			
Forward primer	5' CKACAAAAYCCYTCRAATTG 3' [21]	58° C	58°-1 min, 72°-1 min, 72°-5 min 4°∞
Reverse primer	5' CACCCRGATTYGAACTGGGG 3' [21]		
rbcl			
Forward primer	5' ATGTCACCACAAACAGAGACTAAAGC 3' [8]	58° C	
Reverse primer	5' GTAAAATCAAGTCCACCRCG 3' [8]		
ITS			
Forward primer	5' TCCGTAGGTGAACCTGCGG 3' [22]	58° C	
Reverse primer	5' TCCTCCGCTTATTGATATGC 3' [22]		
matK			
Forward primer	5' CGATCTATTCATTCAATATTTC 3' [23]	54° C	95°-5 min, 94° – 1 min, 54° – 1 min, } 35 Cycles 72°-1 min, 72°-5 min 4°∞
Reverse primer	5' TCTAGCACACGAAAGTCGAAGT 3' [23]		

met the requirements for successful sequencing. The five DNA barcodes *psbA-trnHf*, *RpoB-trnCGAR*, *rbcl*, *ITS*, and *matK* were efficiently amplified using the primers employed; as a result, agarose gel electrophoresis revealed a sharp DNA band, which, when purified, produced successful sequencing results. The identified ten nucleotide sequences for *W. coagulans* have all been submitted to the NCBI Gene Bank database. All DNA markers produced different base-pair lengths with indels/deletions were found after distance and phylogenetic analysis.

3.2. DNA Sequence Data Analysis

NCBI BLAST analysis of all five DNA barcodes obtained from *W. coagulans* (sample 1 and sample 2) with the retrieved sequences of some *Solanaceae* family taxa was aligned, including other *Withania* species. *Ipomoea carnea, Ipomoea batatas,* and *Convolvulus arvensis* were used as out group taxa. *W. coagulans* sample 1 and sample 2 comparative studies is noted [Table 3], and *W. coagulans* sample 1 and sample 2 comparative studies [Table 4] with other sequences retrieved from NCBI databases is also analyzed.

The *psbA-trnHf* gene sequences from *W. coagulans* samples 1 and 2 (WCNB1 and WCNB2), respectively, showed the least amount of length variation (24 bp) and the lowest amount of (G+C) content (27%), in comparison to *matK, ITS, rpoB,* and *psbA* [Table 5], which did not exhibit any pairwise distance average mean. Sequences found 509 conserved sites out of 525 with no variables, parsimony informative sites, and singleton base. The aligned sequences of different species showed base replacement according to statistical analysis of nucleotide pair frequencies, and the transitional rate 14 was lower than the transversional rate 18 in *psbA*. It was discovered that *psbA* has the best ability to distinguish between *Solanaceae* species.

RpoB-trnCGAR gene sequences from *W. coagulans* WCNB1 and WCNB2 (549 bp and 385 bp) showed significant length variation of 164 base pairs and 41.8% and 41.5% (G+C) content, respectively, with 0.132 pairwise distance average mean. Sequences found 327 conserved sites out of 376 base pairs with 44 variables. After alignment, WCNB1 and WCNB2 showed changes in the sequences from positions 1 to 7, and different gaps were found between positions 13 and 39. Purines replaced by purines predominated over purines replaced by pyrimidines when selected sequences were aligned for analysis.

Rbcl was found to be a highly conserved genetic marker in the *Solanaceae* family. The species *Iponeace carnea* showed gaps from positions 1-303; therefore, except for the out-group species *Ipomea carnea*, all 15 taxa were chosen for sequence alignment of the *rbcl* gene exhibited 100% similarity in their sequences. Nonetheless, the sequence from position 303 to 507 for *Ipomoea carnea* showed perfect similarity with other chosen taxa, demonstrating that no insertions or deletions were found in this genetic marker.

The following barcode study used the *ITS* gene sequences from samples 1 and 2 (695 and 696 bp, respectively), with a one base pair variance and 66.9% and 62.6% (G+C) content in each, which is the highest GC content among all the DNA barcodes selected for studies. After *ITS* was aligned, it was found that WCNB1 and WCNB2 had a variety of gaps from positions 50 to 62, 106 to 112, 23 to 34, 47 to 82, 503 to 504, 532 to 534, 600 to 603, 637 to 638, 649 to 653, and 676 to 686. In addition, four single nucleotide gaps were found at locations 144, 164, 173, and 205. In the statistical analysis of nucleotide pair frequencies, transitional pairings 37 are more numerous than transversional pairs 21.

MatK gene sequence lengths for samples 1 (805 bp) and 2 (783 bp), with 22bp variation and 33.3% (G+C) content and 32.8% (G+C) content, for sample 1 and 2 respectively. In the *Solanaceae* family,

Table 2: GenBank accession numbers of submitted sequences of *Withania coagulans* in NCBI [24].

Species name	Accession number of *psbA*-trnHf	Accession number of *rpoB*-trnCGAR	Accession number of *rbcl*	Accession number of *ITS*	Accession number of *matK*
Withania coagulans sample 1 (WCNB1)	OQ406250	OQ406252	ON369434	MW205772	MW729349
Withania coagulans sample 2 (WCNB2)	OQ406251	OQ406253	0N369435	MW205773	MW729350

Table 3: Sequence length and (G + C) % content of barcode regions in *Withania coagulans* species [14].

Species name	*psbA*-trnHf		*rpoB*-trnCGAR		*rbcl*		*ITS*		*matK*	
	Length (bp)	%GC	Length (bp)	%GC	Length (bp)	%GC	Length (bp)	%GC	Length (bp)	% GC
Withania coagulans WCNB1	525	27.5	549	41.8	684	44	695	66.9	805	33.3
Withania coagulans WCNB2	549	27.5	385	41.5	546	44	696	62.6	783	32.8

Table 4: Characteristic features of five DNA markers among *Withania coagulans* sample 1 and sample 2 [14].

Withania coagulans sample 1 and sample 2	Pairwise distance average mean	Number of conserved sites	Number of variables	Number of parsimony informative sites	Number of singletons
psbA-trnHf	0.000	509/525	None	None	None
rpoB-trnCGAR	0.132	327/376	44/376	None	None
Rbcl	0.000	507/507	None	None	None
ITS	0.041	650/744	32/744	None	None
matK	0.029	739/767	22/767	None	None

matK also demonstrated a high level of conservation with 27 single gaps in the sequences of all chosen taxa. Furthermore, in *matK*, the transitional results revealed 22 base pair variations using multiple sequence alignment in both samples of *W. coagulans.*

3.3. Phylogenetic Analysis

A phylogenetic tree was constructed with Maximum Likelihood analysis using Mega 11 software. It showed a higher resolution for all five DNA barcodes. DNA marker sequences for *W. coagulans* (sample 1 and 2) were collected, and a BLAST search was conducted. The selected sequences showed ≥90% similarities from the *Solanaceae* family and out-group taxa were then aligned in MEGA 11 using multiple sequence comparison by Log-Expectation. Using the Maximum Likelihood approach with 1000 bootstrap replications and the Tamura-Nei model, the nucleotide sequences of *W. coagulans* were utilized for evolutionary studies.

In contrast to other species of the *Solanaceae* family, the phylogenetic analysis of *psbA* [Figure 1] shows that it successfully recognizes *W. coagulans*. The *W. coagulans* sample 1(WCNB1) and sample 2 (WCNB2) had the same branch length of 0.00004 and originated from the same branch point belonging to a monophyletic group. All *Withania* species exhibit the most significant degree of similarity. *Ipomoea carnea, Ipomoea batatas,* and *Convolvulus arvensis* are out-group taxa belonging to a separate clade showing another monophyletic cluster with a branch length of 0.01950, which aids in establishing a distinction between the species of the *Solanaceae* family and the out-group taxa chosen. The tree with the highest log probability (–1439.68) is presented.

Phylogenetic analysis of *rpoB* [Figure 2] *W. coagulans* samples 1 and 2 was shown to be in two distinct clusters with branch points of 0.006727 and 0.072480, respectively, indicating a significant difference. The tree with the most excellent log probability (–889.38) is displayed. *W. somnifera* species are most similar to *W. coagulans* sample 1. Whereas *W. coagulans* sample 2 revealed a high similarity with the NCBI-retrieved *W. coagulans rpoB* gene sequence. The three out-group taxa were present in two separate monophyletic clades in the middle of the tree. To distinguish between various *Withania* species, it was found that *rpoB* as a DNA marker was insufficient.

Phylogenetic evaluation of *rbcl* [Figure 3] nucleotide sequences showed a well-resolved tree with multiple clades, whereas *W. coagulans* both samples belonged to a monophyletic group. In contrast, other *Withania*

Table 5: Characteristic features of five DNA markers among *Solanaceae* species and out-group taxa [14].

Solanaceae species and out-group taxa	Number of taxa selected	Pairwise distance average mean	Number of conserved sites	Number of variables	Number of parsimony informative sites	Number of singletons
psbA-trnHf	14	0.135	368/525	149/525	59/525	89/525
rpoB-trnCGAR	10	0.033	305/376	71/376	60/376	11/376
Rbcl	15	0.028	469/507	38/507	33/507	05/507
ITS	14	0.122	488/744	224/744	111/744	109/744
matK	16	0.071	625/767	142/767	110/767	32/767

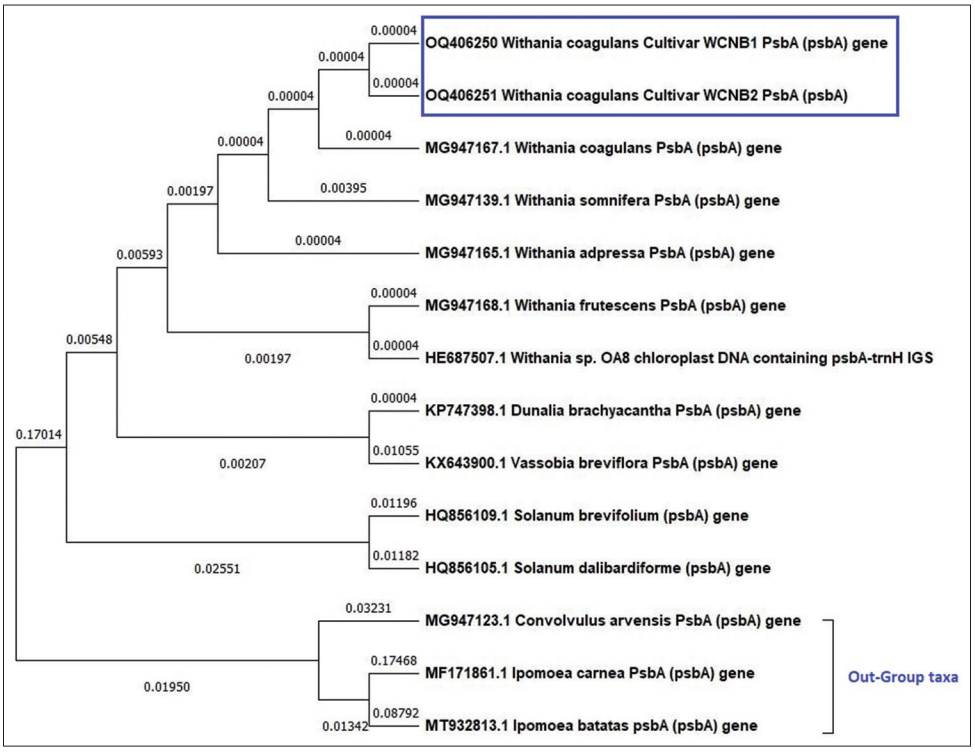

Figure 1: Maximum likelihood phylogenetic analysis using *psbA* genetic marker among *Withania coagulans* samples (WCNB1 and WCNB2), *Solanaceae* species, and out-group taxa.

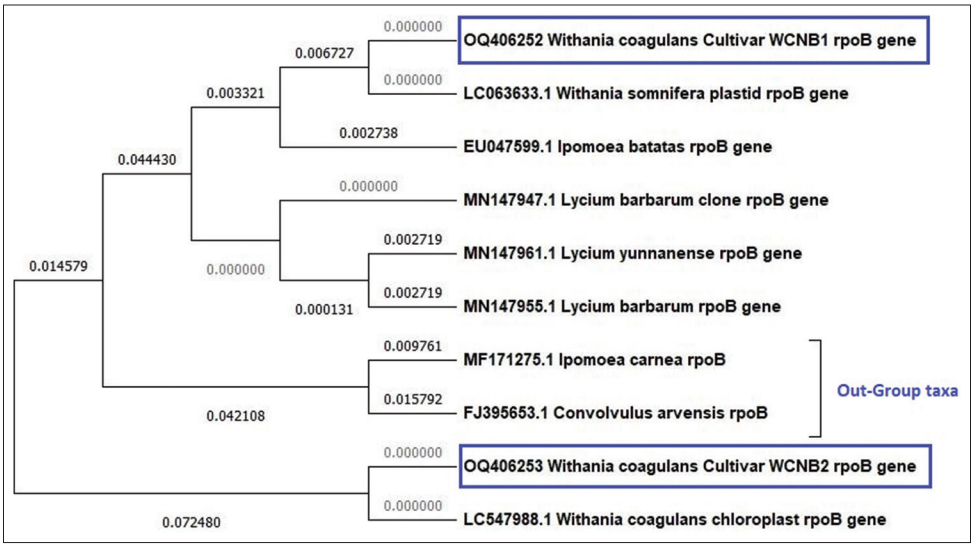

Figure 2: Maximum likelihood phylogenetic analysis using *rpoB* genetic marker among *Withania coagulans* samples (WCNB1 and WCNB2), *Solanaceae* species, and out-group taxa.

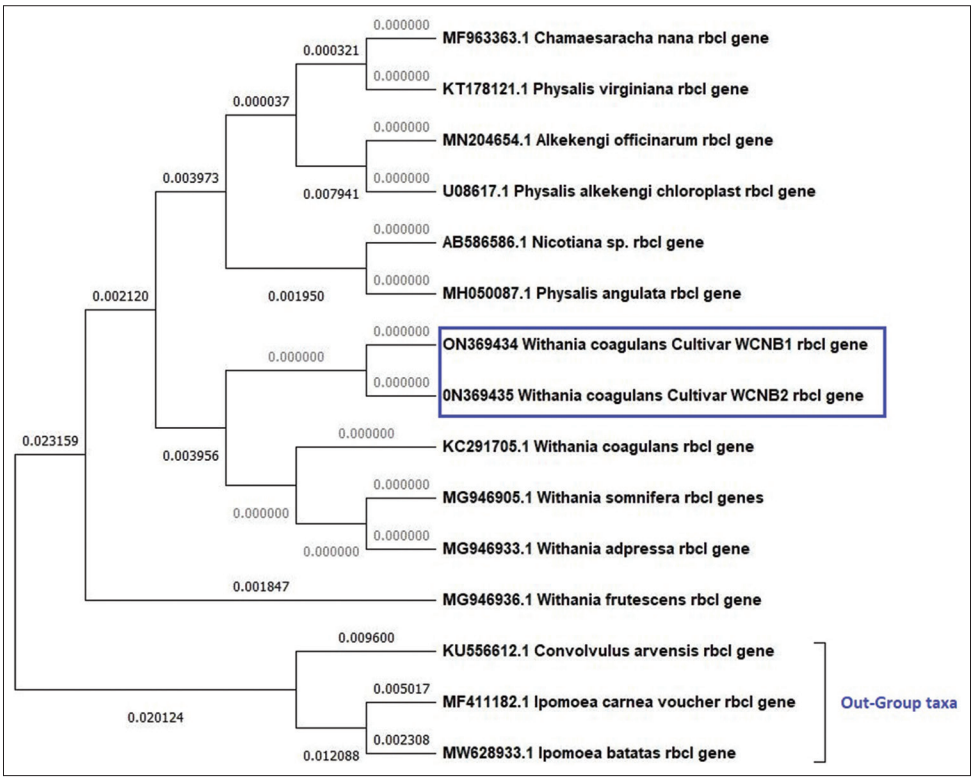

Figure 3: Maximum likelihood phylogenetic analysis using *rbcl* genetic marker among *Withania coagulans* samples (WCNB1 and WCNB2), *Solanaceae* species, and out-group taxa.

species belonged to a separate cluster, including *adpressa*, *frutescence*, and *somnifera* from the same branch point, showing higher similarities in the genus. Furthermore, the out-group displayed a different cluster. Finally, the tree with the most significant log probability is displayed (–967.83).

ITS phylogenetic analysis [Figure 4] revealed, *W. coagulans* WCNB1 and WCNB2 belonged to a non-monophyletic sister clade below clade 1, representing *W. somnifera* sequences, showing exact ancestral origin. The sequence divergences of the *ITS* marker differentiate due to the tree making different clusters in the *Solanaceae* family and the out-group taxa *Ipomoea carnea* and *Convolvulus arvensis* displaying a separate group. The tree was shown with the highest log likelihood (–2621.07).

The phylogenetic analysis of the *matK* sequences [Figure 5] showed a tree with just two clusters, the first of which encompassed all the *Solanaceae* species, including *coagulans* and *frutescens*, the out-group of which formed a monophyletic cluster. The fact that WCNB1 and WCNB2 are clearly distinctly located in the first cluster causes them to exhibit divergences. The tree with the highest log likelihood (–1740.62) is shown.

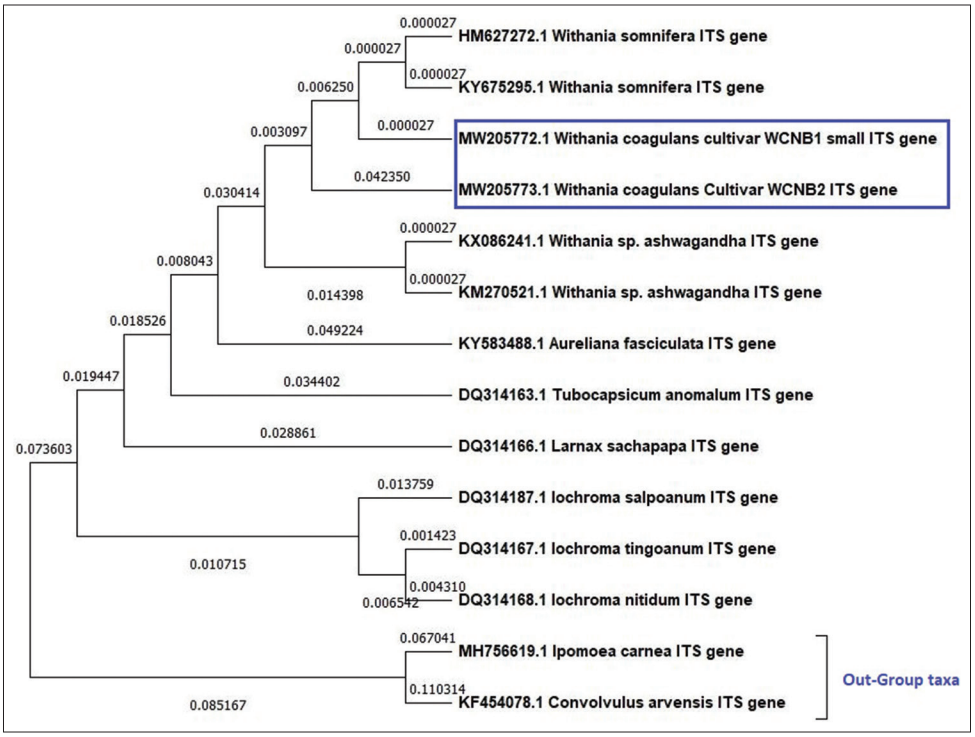

Figure 4: Maximum likelihood phylogenetic analysis using *ITS* genetic marker among *Withania coagulans* samples (WCNB1 & WCNB2), *Solanaceae* species, and out-group taxa.

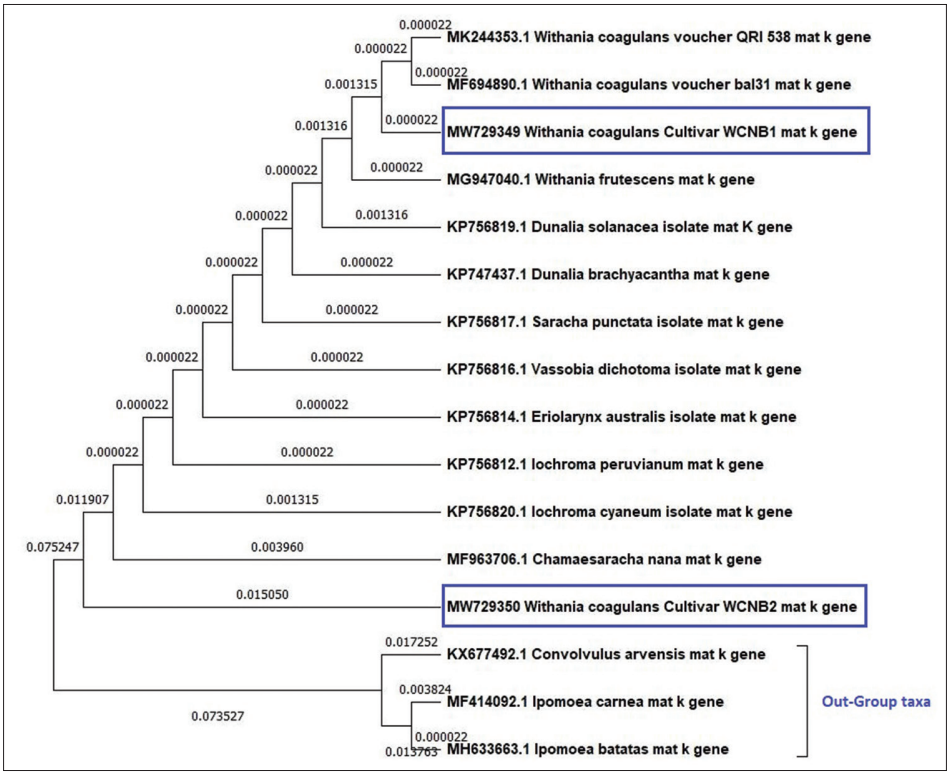

Figure 5: Maximum likelihood phylogenetic analysis using *matK* genetic marker among *Withania coagulans* samples (WCNB1 and WCNB2), *Solanaceae* species, and out-group taxa.

4. DISCUSSION

Understanding the evolutionary history of many significant plants depends on accurately identifying and assessing biologically significant plant species and their families [12]. DNA barcoding soon became a reliable method for identifying species, spotting adulteration, quality control, and ecological assessments. However, it cannot replace the conventional taxonomic identification technique,

but it will be easier to understand phylogeny and its origins with the help of this information. The purity of the DNA is crucial for PCR-based amplification. Even closely related species may need distinct DNA isolation procedures because metabolites in medicinal plants can occasionally affect DNA integrity during isolation [15]. The main principle of plant species identification is the sequencing variation from the reference sequence and phylogenetic reconstruction [16]. To barcode the *W. coagulans* plant in the present study, we used the chloroplast *trnH-psbA*, *rpoB*, *rbcl*, *ITS*, and *matK* region. The chloroplast genomes of angiosperms contain characteristics conserved across and among many plant lineages and have almost identical gene content and organization [17–19]. The amplification and sequencing rates have been high enough to be considered for five selected genetic loci as DNA barcodes.

In the present study, multiple sequence alignment and phylogenetic analysis showed that compared to the other three possible barcodes, *rbcl*, and *psbA* sequences have a low rate of nucleotide evolution due to the presence of highly conserved regions in them. In both the WCNB1 and WCNB2 samples, *rbcl* demonstrated complete conservation. Furthermore, compared to the *Solanaceae* family species, it showed the most significant similarity. However, the out-group taxa had some differences and belonged to a different group on the phylogenetic tree. With an average mean distance of 0.000 between WCNB1 and WCNB2, *psbA* demonstrated significant levels of conservation and the highest similarity among the *W. coagulans* species, *matK* sequence analysis revealed 22 variable sites for *W. coagulans*, and *rpoB* showed the highest number of 44 variable sites.

The best genetic markers for identifying the *W. coagulans* plant were *psbA* and *rbcl*. It revealed no genetic differences even when samples were grown in ICAR-Gujarat, and The Institute of Science, Mumbai. However, *rpoB*, *ITS*, and *matK* differed in both samples due to 22-44 bp variations. The results of this work indicate that the above-mentioned indigenous plant species can be amplified, identified, and differentiated successfully using universal primers (*rbcl+psbA*) for DNA barcoding. "*matK* can also be used due to fewer variations in the evolution of genes. Over 90% of flowering plants can be distinguished using the (*rbcl+matK*) pair combination," according to CBOL 5. However, more conserved genomic areas could find and used to identify adulteration of therapeutic material. Once the barcodes have evaluated, the barcode markers *psbA*, *rbcl*, and *matK* can be combined to create a barcode appropriate for identifying *W. coagulans*. *W. coagulans* samples can be separated using the present study's (*rbcl+psbA)* combination.

As per the Log-likelihood values by MEGA 11 in the phylogenetic analysis, it was observed that the *rpoB* marker showed the highest probability value (–889.38) than *rbcl* (-967.83). The other three markers showed the lowest values. Hence, it was proved to have the *rpoB* gene with fewer possible mutations in the evolutionary studies.

5. CONCLUSION

The current work examined two samples of *W. coagulans* produced at ICAR-Anand and the Institute of Science, Mumbai for potential evolutionary relationships using modern DNA barcode technologies and five genetic markers. This research is the first to evaluate molecular markers based on identifying and classifying *W. coagulans* from two sites in India cultivated from the same seed samples. The current work uses DNA barcoding techniques and offers essential results for establishing the phylogeny and relationship between several *Solanaceae* family species. Genetic markers *rbcl* and *psbA* show the highest conservations, whereas *matK*, *ITS*, and *rpoB* can be discriminating markers in the *Withania* genus and the *Solanaceae* family. More DNA barcode investigations are required to describe *W. coagulans* from all possible growth-friendly environments. This research will help gather basic information and provide details on the species molecular taxonomy.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This research does not involve any animal or human subjects for ethical approvals.

10. DATA AVAILABILITY

This research publication includes all data gathered and analyzed, and the sequences are available online in the NCBI database.

11. PUBLISHER'S NOTE

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