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DNA barcode based cultivar authentication in *Clitoria ternatea* L. using rpoB and rbcL conserve regions

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Abstract

Clitoria ternatea (*C. ternatea*), generally known as Shankhpushpi, is an essential substance in Ayurvedic medicine used for memory enhancement, and as a nootropic, tranquillizing, anti-stress, anxiolytic, anti-convulsant and sedative agent. Identification of the vegetative state of cultivars is a challenging action within the species. In the current study, 6 cultivars were recognized by using universally conserved sequences. DNA barcoding is based on molecular markers technology, typically used for diversity or species identification. To investigate 6 distinct *C. ternatea* L. varieties that were collected in and around Bhubaneswar, Odisha, we employed 9 conserved locus barcode locations: atpF-atpH, rpoB, matK, rpoC, psbK-psbI, trnH-psbA and rbcL. After PCR, gel electrophoresis yielded significantly distinct bands for 5 markers: rbcL, rpoB, atpF-atpH, trnH-psbA and psbK-psbI. Phylogenetic analysis and sequencing of the amplified bands using the rpoB and rbcL barcode primers revealed insertion and deletion, which were suggestive of consistent separation of all 6 cultivars. Consequently, these markers may be utilized to identify the cultivars of *C. ternatea*. These barcode markers may also be used to recognize difference in *C. ternatea* cultivars.

Keywords

DNA barcode; Clitoria ternatea; rpoB; rbcL; SNPs; insertion; deletion

Introduction

The butterfly pea, or *Clitoria ternatea* L. (*C. ternatea* L.), is a leguminous plant native to Asia that belongs to the Fabaceae family and is prized for its vivid blue flowers. Due to its strong antioxidant content, the plant is frequently used as a natural color and in herbal teas. The plant is a perennial herbaceous climber with elliptical, blunt leaves. *C. ternatea* has slender stems, pinnate leaves and solitary blue-to-blue mauve flowers. Its pods are flat, elongated and have a pointed tip. The pods can reach lengths of 6–12 cm and width of 0.7–1.2 cm. They contain up to 10 seeds, which can be olive, brown, or black and have a mottled pattern. The seeds have an average length of 4.5–7 mm and a width of 3–4 mm (1). *C. ternatea* has enormous deep-root mechanisms that allow it to tolerate droughts lasting up to 7–8 months. The morphological classification is shown in Table 1. This plant is primarily found in tropical areas that need intense sunlight and tolerate some degree of abiotic stress. The roots of legume plants form nodules that help in nitrogen fixation and provide a natural fertilizer for agricultural land (2).

Table 1. The morphological structure of several cultivars of *C. ternatea*

Cultivar name morphological identification	White *C. ternatea* F Albiflora single	White *C. ternatea* F Albiflora double	Blue *C. ternatea* single	Blue *C. ternatea* double	Light pink *C. ternatea* single	*C.fairchildiana*
Flower colour	Pure white	Pure white	Deep blue with a characteristic white or yellowish throat	Vibrant blue	Light pink, generally with a faint, somewhat darker pink or white centre	Deep violet to purple-blue in color
Average leaf length	4-6 cm	5-7 cm	3-5 cm	5-7 cm	4-6 cm	5-7 cm
Petal arrangement	Standard papilionaceous flower	Standard papilionaceous flower and double form modifies this typical structure with extra petals.	Flowers are papilionaceous	Papilionaceous flower characteristic, petals modified by additional petal layers in the double form.	Standard papilionaceous flower	Flowers have a standard papilionaceous structure with five petals
Seed number	6-10					4-6
Seed size	0.5-0.8 cm	0.4-0.5 cm	0.4-0.5 mm	0.4-0.5 mm	0.5-0.8 cm	0.4-0.5 cm
Flower symmetry	Bilateral symmetry					

It has recently gained significant attention due to its potential applications in modern agricultural and medical fields and its use as an antioxidant and a natural source of food pigments (3). This plant has many cultivars, such as *Clitoria fairchildiana*, blue *C. ternatea* single, blue *C. ternatea* double, light pink *C. ternatea* single, white *C. ternatea* F Albiflora single and white *C. ternatea* F Albiflora double with a distinct bloom shape and limited developmental structure (4). Therefore, it is a great challenge to identify the cultivar in the species in a vegetative state.

Various flora species management and preservation programs developed due to climate changes in the last few years have advocated the clear understanding of the genetic structure of species at the population level (5). The presence of distinct genetic characteristics separates individuals of one community from those of another. A population with a high degree of diversity is more adaptive and hence, more fit in continually changing surroundings (6). It is possible to predict how populations will respond to future events of both natural and artificial origins by evaluating population genetic structure. The population genetic structure also provides a historical picture of changes in evolution that define a species. Due to changes in gene expression or encoded protein activity involved in secondary metabolic pathways brought about by various stresses, the secondary metabolite content of medicinal plants with the same genetic background may vary during developmental processes or in response to various stresses. Hence, novel techniques from the fields of genomics, transcriptomics, and metabolomics will have to be employed to acquire better knowledge about the spatial and temporal patterns of active ingredient synthesis and accumulation in certain plant organs, tissues and cells (7). The studies on genetic diversity and bioactive metabolite estimation are necessary for the conservation of *C. ternatea*. This will help in the long term while selecting a genotype and developing pure line genotypes with higher concentrations of desired bioactive metabolites and plant types to fulfil the needs of the herbal medicine and floral cultivation industries, respectively (8). The genetic resource of *C. ternatea* is also crucial for the production of a variety of bioactive metabolites, such as flavanol glycosides, kaempferol, quercetin, taraxerol, anthocyanins, cyclotides, triterpenoids and lactones (9). These components have anti-cancer (10), anti-diabetic (11), antioxidant (12), anti-bacterial (12), anti-inflammatory (13) and analgesic properties (14), along with other roles as memory enhancers (15), pain relievers (16), anxiety-reducing drugs (17), tranquilizers (18) and sedatives (19). Genomic barcoding allows for the accurate and quick identification of species variety in intricate biological ecosystems by analysing the sequence of standardised gene sections. Standardised short DNA sequences (~400–800 base pairs) are utilised as "barcodes" in this approach to identify recognised species and evaluate biodiversity (20). Molecular marker-based DNA barcoding is a common technique for identifying species or variations. It is now widely used in evaluating and documenting biological resources and has proven to be an effective method for identifying species (21). It requires the use of a number of plant-conserved chloroplast regions, matK, atpF-atpH, rbcL, trnH-psbA, psbK-psbI and around ~650 bp of the mitochondrial cytochrome oxidase 1 (COI) region in animals (22). Effective species identification using DNA barcodes depends on the ability to consistently detect a substantial difference between the average genetic distances within a species and between different species. Bio-conservation, systematics, forensic investigation and ecology have recently been highly interested in DNA barcoding, which typically requires a tiny and recoverable DNA fragment (23). The accurate identification of species, the discovery of traditional knowledge about the use of these plants, the preservation of this important and endangered ethnomedical plant bioresource and the improvement of these plants via biological prospecting are necessary. This study investigated 6 different varieties of *C. ternatea* collected from the surrounding area of Bhubaneswar, Odisha. The analyses focused on 9 conserved locus barcode sections (rbcL, psbK-psbI, rpoC, rpoB, matK, atpF-atpH and trnH-psbA). There is scope for the identification of suitable DNA identifiers and any SNPs that may be uniquely associated with a specific variety.

Materials and Methods

Sample collection

Fresh (disease-free) and immature leaves of all 6 *C. ternatea* cultivars (depicted in Fig. 1 and Table 2) were gathered from various locations around Bhubaneswar, Odisha with the permission of local authorities from the Regional Plant Research Center, Odisha. The plant was taxonomically identified and authenticated by Dr. Laxmikanta Acharya (Professor, Centre for Biotechnology, Siksha 'O' Anusandhan University, Odisha, India) and a voucher specimen (SOAU/CBT/2024/ER/08) was retained in the department for future reference and the plant has been maintained in an environmentally controlled greenhouse. Experimental research on the plant used for the study complies with relevant institutional, national and international guidelines and legislation.

DNA isolation

The genomic DNA was isolated from the leaves using the Doyle & Doyle DNA isolation procedure (11). The purified DNA was quantified by comparing the standard λ DNA band in a 0.8% agarose gel to an ethidium bromide (EtBr) dyed sample DNA band. DNA samples were diluted with nuclease free water to achieve a 50 ng/µL working concentration. The sequences for the barcode primers were obtained again from the BOLD systems database. Designed primers were used in PCR amplification (Table 3).

PCR amplification

The Veriti 96 well and GeneAmp 9700 thermo cyclers (Applied BioSystems), were utilized in this application. A reaction volume of 25 µL was used and amplification was done using the following reaction conditions: initial denaturation at 96°C for 5 min, followed by 35 cycles of denaturation at 92°C for 1 min, incubation at annealing temperature (which varied based on the primer set) for 1 min and amplification at 72°C for 2 min and a final extension step at 72°C for 7 min. The amplified PCR products were examined on 1.5% agarose gel after electrophoresis (Fig. 2 and3).

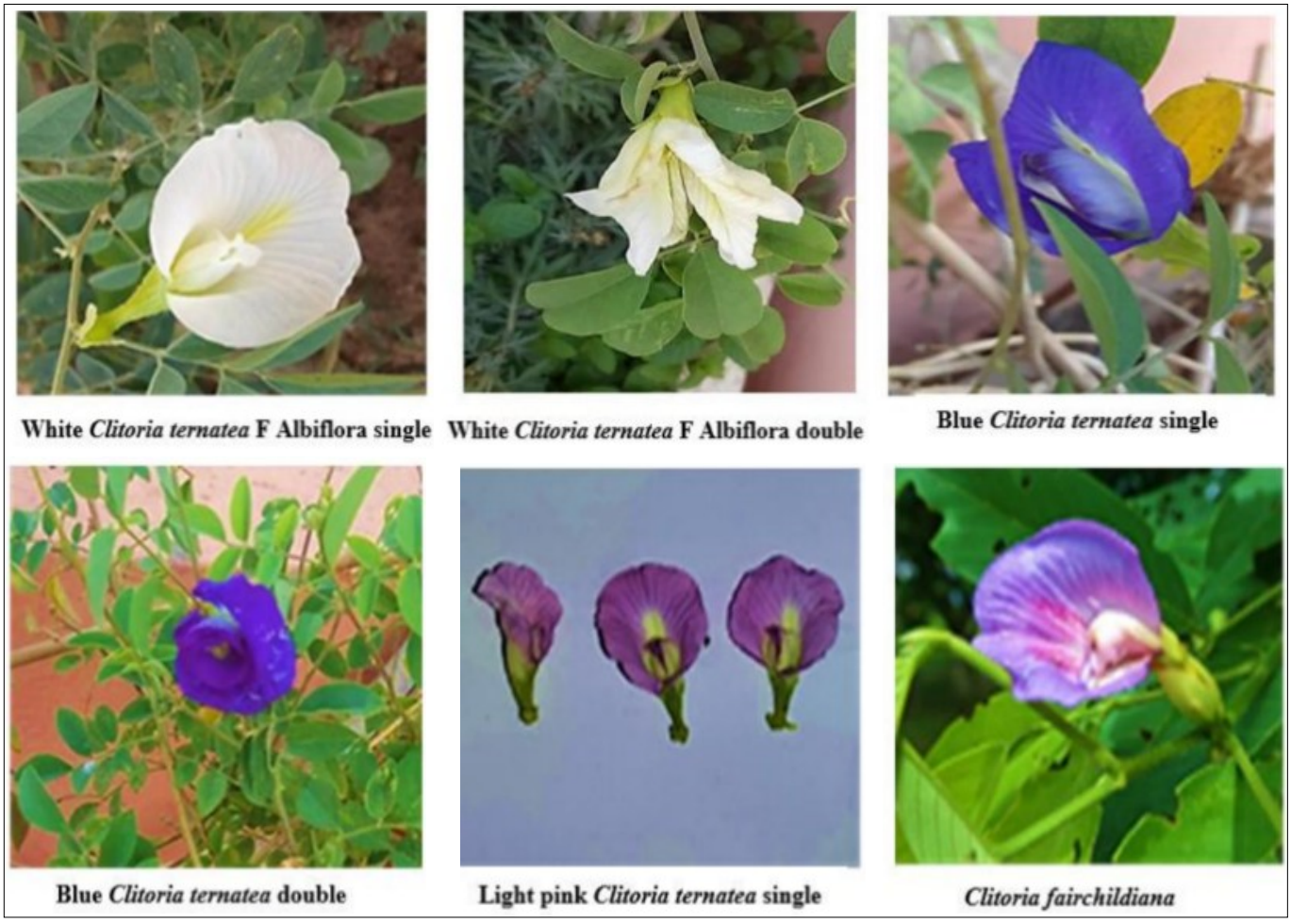

Fig. 1. Distinctive *C. ternatea* cultivars gathered from various regions of Odisha.

Table 2. Different cultivars of *C. ternatea*

Cultivar	Cultivar name	Code
1	White *Clitoria ternatea* F Albiflora single	AS1
2	White *Clitoria ternatea* F Albiflora double	AS2
3	Blue *Clitoria ternatea* single	AS3
4	Blue *Clitoria ternatea* double	AS4
5	Light pink *Clitoria ternatea* single	AS5
6	*Clitoria fairchildiana*	AS6

DNA sequencing and PCR product purification

The amplified bands achieved from PCR reactions using a pair of primers set (LKB 1–2, LKB3–4 and LKB 15–16) yielded visible bands of expected size. These bands were extracted using the Silica Bead DNA Gel Extraction Kit while

Table 3. Barcode primers have been obtained from the Barcode of Life Database (www.barcodinglife.org.)

Locus	Primer name	Primer sequence (5'-3')	Annealing temperature (in°C)	Fragment range (in bp)
rpoC1	LKB1	GGCAAAGAGGGAAGATTTCG	49	514-575
	LKB2	CCATAAGCATATCTTGAGTTGG		
rpoB	LKB3	ATGCAACGTCAAGCAGTTCC	49	428-531
	LKB4	CCGTATGTGAAAAGAAGTATA		
	LKB5	CGTACAGTACTTTTGTGTTTACGAG	53	741-868
	LKB6	ACCCAGTCCATCTGGAAATCTTGGTTC		
matK	LKB7	TCTAGCACACGAAAGTCGAAGT	49	858-963
	LKB8	CGATCTATTCATTCAATATTTC		
	LKB9	GTTCTAGCACAAGAAAGTCG	45	941-1154
	LKB10	TAATTTACGATCAATTCATTC		
atpF-atpH	LKB11	ACTCGCACACACTCCCTTTCC	53	611-827
	LKB12	GCTTTTATGGAAGCTTTAACAAT		
psbK-psbL	LKB13	TTAGCCTTTGTTTGGCAAG	49	418-427
	LKB14	AGAGTTTGAGAGTAAGCAT		
rbcL	LKB15	GTAAAATCAAGTCCACCRCG	53	500-581
	LKB16	ATGTCACCACAAACAGAGACTAAAGC		
TrnH-psbA	LKB17	GTTATGCATGAACGTAATGCTC	53	279-500
	LKB18	CGCGCATGGTGGATTCACAAATCC		

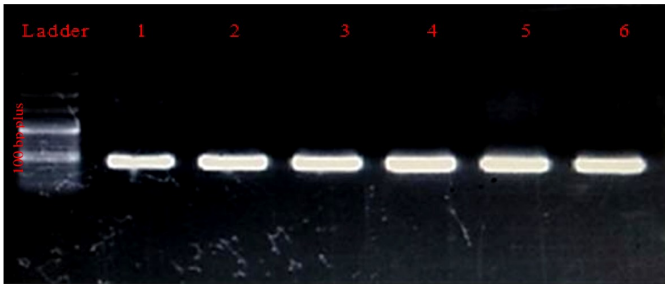

Fig. 2. Gel image shows fare, distinct bands (~514 bp) for the rpoB "bar-code primer" of six cultivars of *C. ternatea.*

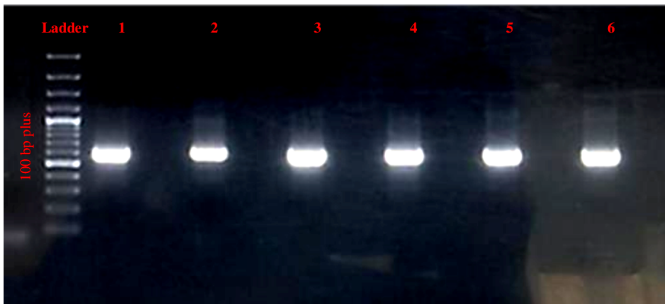

Fig. 3. Gel image shows clear, distinct bands (~550 bp) for the rbcL barcode primer of six cultivars of *C. ternatea.*

adhering to the below-mentioned technique. A scalpel blade was used to cut the desired DNA bands from the agarose gel while the gel was viewed on a UV transilluminator. The gel pieces were transferred to several pre-weighed micro-centrifuge tubes and weights of the gel pieces were recorded. 6M NaI solution was added to the micro-centrifuge tubes holding the gel fragments in an amount equal to three times the weight of the gel fragments. The tubes were incubated in a water bath set at 55 °C for 10-15 min to melt the gel fragments. Once molten, 10 µL of silica gel was added to each tube before centrifuging at ambient temperature for 1 min at 10000 rpm. The supernatant was removed from each micro-centrifuge tube, the pellet was resuspended in 1 mL of an ultra-clean solution (5M NaCl, 1M Tris-Cl, 0.5M EDTA, 95% ethanol, double-distilled water) The tubes were again centrifuges for 1 min at 10000 rpm at ambient temperature. After discarding the supernatant, the centrifugation procedure was repeated with the addition of an ultraclean solution. After discarding the supernatants, 50 µL of nuclease free water was added to each micro-centrifuge tube, mixed by gentle tapping and incubated at room temperature for 5–10 min. The tubes were centrifuged at 10000 rpm for 1 min. The supernatant was retrieved into new micro-centrifuge tubes and pellet was discarded. The supernatants were used for sequencing on an ABI3730XL platform using the Sanger sequencing technique. The appropriate forward and reverse primers were applied for both forward and backwards sequencing.

Sequence analyses

The CAP3 Sequence Assembly Program (PRABI-Doua) created consensus sequences by clipping the retrieved sequences (https://doua.prabi.fr/software/cap3). The sequences found for rpoB (LKB 3-4) and rbcL (LKB 15-16) were around 514 and 550 bp, respectively. Expasy Translate Tool (https://web.expasy.org/translate) was used to identify the correct amino acid sequences and ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder) was used to confirm them. The Clustal X program was used to independently align the rpoB and rbcL sequences for the combined set of data. Both intra-species and inter-species divergence were calculated using the CLUSTAL multiple sequence alignment method using the MUSCLE (3.8) technique (Fig. 4 and 5).

Fig. 4. The alignment of 6 sequences of rpoB of *C. ternatea*, including In/Dels and SNPs from different locations.

Phylogenetic analyses

Divergences in pairwise nucleotide sequence were used to create the CLUSTAL multiple sequence alignment by MUSCLE (3.8) and the distance matrices in MEGA 11 to investigate the phylogenetic relationships between the five different cultivars of the investigated species of *Clitoria*. It was completed for the rpoB and rbcL sets.

Results

Sequence analyses

The different single nucleotide polymorphisms (SNPs); and insertion and deletions (In/Dels) in the rpoB and rbcL barcodes amongst the 6 cultivars is presented in Fig 4. Comparing white *C. ternatea* F Albiflora single and white *C. ternatea* F Albiflora double, we have observed an intri-

Fig. 5. The alignment of 6 sequences of rbcL of *C. ternatea*, including In/Dels and SNPs from different locations.

guing characteristic in the location of In/Dels and SNPs. The SNPs and In/Dels were similar for both taxa. However, blue *C. ternatea* single and blue *C. ternatea* double are identical to each other compared to light pink *C. ternatea* single. We observed a unique characteristic in the location of In/Dels for *C. fairchildiana*. It has the highest number of

SNPs and also In/Dels as compared to all other cultivars (Table 4). In the case of rpoB, the lowest average nucleotide sequence was found in *C. fairchildiana* i.e., 1.76 after SNP and 19.95 after insertion and deletion. Similarly, in rbcL, the lowest average nucleotide sequence was found in *C. fairchildiana* i.e., 6.4 after SNP and 7.40 white *C. ternatea* F Albiflora double after insertion and deletion. Therefore, comparable chloroplast DNA intergenic spacers, rbcL & rpoB, In/Dels and SNPs, may be employed as qualitative molecular markers for studies at the intra-specific and inter-specific levels of the cultivars. Barcodes can be used to identify different plant species' cultivars in addition to their species. As a result, barcode areas may no longer be used exclusively for species identification and can now be used to identify cultivars.

pink *C. ternatea* single, blue *C. ternatea* single and blue *C. ternatea* double. The sample of white *C. ternatea* F Albiflora single formed an independent subclade. In this subclade light pink *C. ternatea* single, blue *C. ternatea* single and blue *C. ternatea* double were much similar than white *C. ternatea* F Albiflora double. Similarly, Fig. 7 shows that it divided all samples and the sequences from the analysis into 1 clade and 4 subclades. The subclade of C. *ternatea* included white *C. ternatea* F Albiflora single, white *C. ternatea* F Albiflora double, light pink *C. ternatea* single, blue *C. ternatea* single and blue *C. ternatea* double. The sample of light pink *C. ternatea* single formed an independent subclade which is unique from all the cultivars. White *C. ternatea* F Albiflora single and white *C. ternatea* F Albiflora double had much more similarity than other cultivars and

Table 4. After multiple sequence alignment, the SNPs and In/Dels that were present in rpoB and rbcL in the five cultivars of *C. ternatea* were identified as follows

SL No.	Cultivar name	SNPs rpoB	In/Del rpoB	SNPs rbcL	In/Del rbcL
1	White *Clitoria ternatea* F Albiflora single	15	30	31	39
2	White *Clitoria ternatea* F Albiflora double	13	29	19	44
3	Blue *Clitoria ternatea* single	23	28	2	8
4	Blue *Clitoria ternatea* double	16	29	2	8
5	Light pink *Clitoria ternatea* single	35	23	13	6
6	*Clitoria fairchildiana*	249	22	90	51

Phylogenetic inference

All of the cultivars were determined to be highly diverse from one another, forming unique groups; the sequences were subjected to phylogenetic analysis and the tree was constructed using the information gathered (Fig. 6 and 7). Fig. 6 shows that *C. fairchildiana* was taken as the outlier from the other five cultivars that had maximum likelihood based on the analyses. It divided all samples and the sequences from the analysis into 1 clade and 3 subclades. The subclade of *C. ternatea* included white *C. ternatea* F Albiflora single, white *C. ternatea* F Albiflora double, light

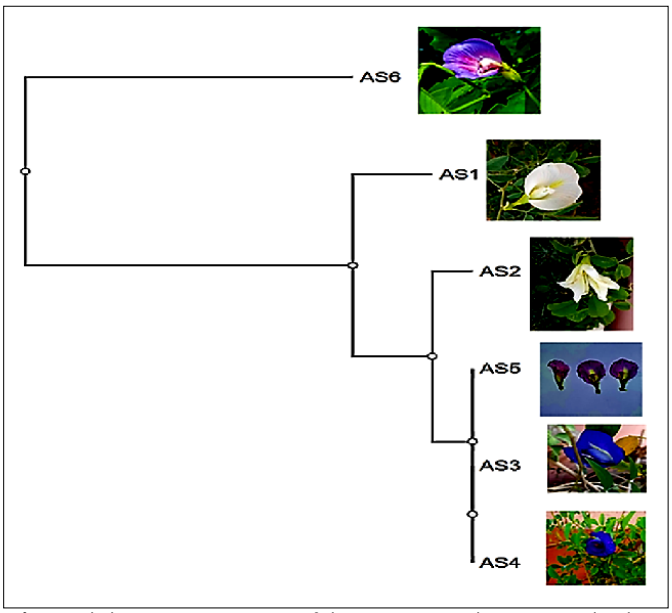

Fig. 6. Phylogenetic conjunction of the *C. ternatea* cultivars using the rbcL barcode primer in the context of a CLUSTAL multiple sequence alignment using MUSCLE (3.8).

also blue *C. ternatea* double had slight similarity to blue *C. ternatea* single forming an independent subclade. Here *C. fairchildiana* was taken as the outlier from the other five cultivars that had maximum likelihood based on the analyses.

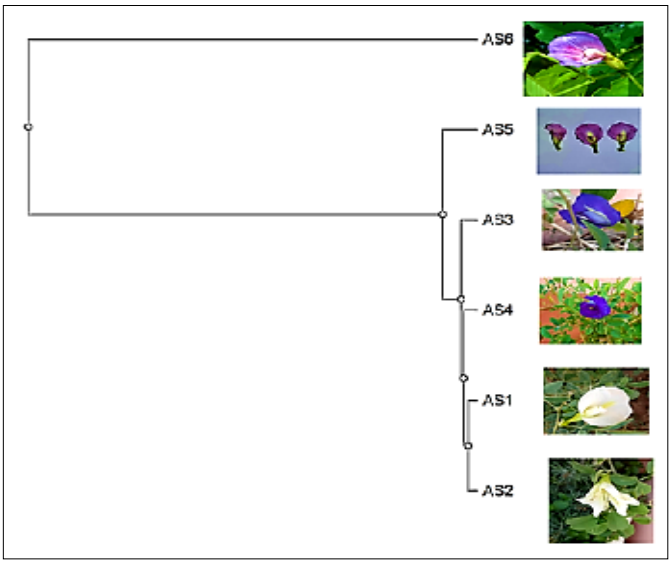

Fig. 7. Phylogenetic conjunction of the *C. ternatea* cultivars using the rpoB barcode primer in the context of a "CLUSTAL multiple sequence alignment" using "MUSCLE (3.8)".

A search of the database was carried out with the use of sequences of the rpoB and rbcL genes that were created independently and contained in the plastid area of the plant specimen. White *C. ternatea* F Albiflora double was the primary identification of the specimen by BLAST search using the single gene sequence of rbcL, with 98.46% sequence similarity. A BLAST search using a single

with 99.77 % sequence similarity, gene rpoB also recognised this specimen as white *C. ternatea* F Albiflora double. The minimum pair-wise rpoB nucleotide sequence similarity was found to be 92.25% (White *C. ternatea* F Albiflora single with *C. fairchildiana*) and a maximum of 98.46% (White *C. ternatea* F Albiflora double with white *C. ternatea* F Albiflora single). On the other hand, the rbcL nucleotide sequences of the examined specimens (White *C. ternatea* F Albiflora double with white *C. ternatea* F Albiflora single) exhibited 99.77% pairwise sequence similarity. The plastid rbcL gene exhibited the lowest average pairwise sequence similarity at 90.04% and differentiated all plant species under all the cultivars of *C. ternatea*. The rbcL nucleotide sequence could not distinguish Blue *C. ternatea* single and blue *C. ternatea* double with light pink *C. ternatea* single due to identical sequence. In contrast, the rpoB nucleotide sequence distinguished all the species.

Discussion

Species identification has become challenging due to the lack of conventional taxonomists and discrepancies in observable characters (24). It is essential to appropriately identify and evaluate biologically significant plant species and their families in order to have a comprehensive understanding of the evolutionary history of several essential plant species (25). DNA barcoding provides data that enhances taxonomy and guarantees accurate organism identification. The sequencing variation from the reference sequence and the phylogenetic reconstruction are the two most important principles that are used in the process of identifying plant species (26). In/Del and unique SNPs may be used to identify species accurately (27). According to the analyses given earlier, 6 sequences for rpoB and 6 sequences for rbcL barcode primer were discovered for each of the 6 cultivars (28). In the current investigation, we applied the chloroplast atpF-atpH, rpoB, matK, rpoC, psbK-psbI, trnH-psbA and rbcL regions in order to barcode the *C. ternatea* cultivars (29). Multiple sequence alignment analyses of the sequences allowed us to get information on the occurrence of single nucleotide polymorphisms (SNPs) and insertion and deletions (In/Dels) in those sequences. In this work, we tested 2 plastids i.e., rbcL and rpoB. Here, rpoB was showing the best performance in PCR amplification and sequencing reactions. However, rbcL had a comparatively low PCR success rate. Therefore, using the conserved areas gathered in the current analysis, it may be able to identify a taxon below its species classification. These sequence-specific regions may be used to create primers that are unique to identifying certain *C. ternatea* cultivars.

Conclusion

In this study, we have investigated the DNA barcode markers that were used to authenticate 6 species of *C. ternatea* cultivars. The barcoding gap and SNPs identified in interspecies and intra-genetic distance can be extremely beneficial for easier identification of the aforementioned taxa. We have taken into consideration a minor proportion of the conserved sections, but sequencing the whole rbcL, rpoB and other barcoding-related genes will allow us to comment further on the outcome. Hence, the DNA barcode marker approach might be helpful in deriving genetic connections of the diverse species in the tribe and will be a promising candidate in resolving taxonomical disputes, determining adulteration in herbal products and identifying the illegally traded species.

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Authors' contributions

SD was involved in drafting and writing the manuscript and in the analyses of work. AS was involved with all the experimental work and LA conceptualized the study, supervised the work, analyses and provided support with reviewing and editing of manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical issues: None

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