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DNA BARCODING OF *ANEMONE OBTUSILOBA* VAR. *POTENTILOIDES*: A THREATENED AND ENDEMIC PLANT FROM THE WESTERN HIMALAYAN REGION OF PAKISTAN

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ABSTRACT

Endemic flora is the bio-assets of a country being medicinally important with economic potential. But unfortunately, these plants are endangered and may become extinct because of their limited geographic distribution and small population size. DNA barcoding is one mong the prime steps for the conservation of a threatened species. *Anemone obtusiloba* var. *potentilloides* (Camb. ex Berant.) Lauener is an endemic plant species belonging to the family Ranunculaceae. The main aim of this study was to find the most effective DNA barcode regions for species identification and authentication. In this study, we used four DNA barcode markers (*matK*, *rbcL, trnH-psbA* and ITS) to explore their effectiveness and efficacy for correct identification of *Anemone obtusiloba* var. *potentilloides*. We examined their efficacy on the bbasisof amplification and sequencing success rate. The data was analysed by BLAST sequence similarity, TAXONDNA, and the Neighbour Joining Tree Method. The universal DNA barcode markers namely *rbcL trnH-psbA* and ITS had shown good amplification and sequence rate while *matK* remained challengeable. After evaluation of the candidate barcodes, only *rbcL* region had shown the highest number of variable sites (1142) as compared to other barcodes. Similarly, *rbcL* came up with the highest score of species identification with 98% and 80.55% in BLAST and TAXONDNA analyses respectively. In cladding analysis, the highest model support was in *rbcL* having the highest bootstrap value as 89% with its relative species. The finding of the current study supports *rbcL* as the best DNA barcode for the identification of *Anemone obtusiloba var. potentilloides*. Moreover, other candidate barcodes were not found efficient for correct identification of the species. This work has useful applications in the field of biodiversity conservation.

Key Words: Endemic flora, *rbcL*, DNA barcode, Phylogenetic analysis, biodiversity conservation.

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INTRODUCTION

Pakistan has a great diversity of plants, honoured by 410 endemic plants of which most plants are confined to the Himalayan region (Khan *et al*., 2019a). Generally, the endemic plants are more susceptible to anthropogenic threats and climatic changes suffering from a high extinction rate making (Coelho *et al.,* 2020). Conservation of the threatened plants is essential for the biological diversity. The first step in the conservation is correct identification and proper delimitation of the target species (Hartvig *et al*., 2015).

Anemone is a genus of the family Ranunculaceae distributed in the temperate zone of the Northern and Southern hemispheres with more than 150 species. Morphologically and phytochemically this genus is closely related to the *Pulsatilla*, *Clematis*, and *Hepatica* (Hao and Xiao *et al*. 2020). In Pakistan, this genus is represented by 7 to 8 species. *Anemone obtusiloba* is represented by two varieties: *Anemone obtusiloba* var. obtusiloba and *Anemone obtusiloba* var. *potentilloides*. Both are endemic to the Western Himalayan region, with *A. obtusiloba var. potentilloides* distributed only in Kashmir and Pakistan (Rajput and Agnihotri, 2020).

DNA Barcoding is a novel and valuable method for identification and discrimination of species. The method can identify even a small and damaged part of the plant species which is difficult to identify morphologically. It uses a small and reliable gene sequence of a standardised portion of a genome. The method is not only used for the identification, but also for investigating the phylogeny, evolution and ecological process (Wang *et al*., 2018). The classification and taxonomy are the fundamental processes for the correct naming of the species. The traditional method of identification is based on morphological characteristics, but often some plants have very similar morphological features making the identification of species very ambiguous. To overcome this problem the molecular method was introduced by Hebert *et al.* (2003); has revolutionized the field of Taxonomy.

In DNA barcoding, a small piece of tissue can be used for the identification, even a highly degraded specimen. In this method, the more closely related species show more homologous DNA sequences, and the distant species have variation in their gene sequences (Gogoi and Bhau, 2018). Besides identifying the target species, DNA barcoding is also a good way to explore floristic knowledge by forming a DNA barcoding reference library of a whole local flora that can be used to analyse all the floristic information quickly without any taxonomic experts (Hartvig *et al*., 2015). Recently, the DNA barcoding of endemic and medicinal flora of Pakistan have been reported and the findings have provided baseline for the use of modern technology for taxonomic studies of wild plants in Pakistan (Khan *et al*., 2019a, b; Umer *et al*., 2020).

The identification and recognition of *Anemone* species is difficult having some morphologically complex features. However, DNA barcoding is usefa ul tool to identify and distinguish the species rapidly and accurately. In a recent study, four commonly used markers namely *trnH-psbA*, *rbcL*, *matK* and ITS were used, among these markers ITS revealed the highest success rate (Li *et al*., 2019). The specific aim of this study is to determine the most useful barcode and make a reference library for Anemone by using the (CBOL) suggested barcodes i.e., *trnH-psbA*, *rbcL*, *matK* and ITS. The study also seeks to test the species delimitation ability of the used barcodes.

MATERIALS AND METHODS

Study Area: The sample of the studied species was collected from Thandiani, a hilly area in Khyber Pakhtunkhwa province, Pakistan. The study area has a great diversity of important plant and animal species. It is found at Northeast of Abbottabad city between 34°-17 to 34°-10 North latitude and 73°-23 to 73°-17 East longitudes with an altitude of 2626 meters (Khan *et al*., 2012). The field work has been conducted during May 2017. The GPS coordinates were recorded in the field. The plant specimen was pressed and left to dry. The collected plant was brought to the Genetics Lab in the Department of Genetics, Hazara University, Mansehra. GPS coordinates are shown in Table 1.

Table 1. Description of the collection.

Species	Family	Location	Latitude	Longitude	Elevation
Anemone obstiloba	Ranunculaceae	Thandiani	34.23	73.35	2626

DNA Extraction, PCR Amplification and Sequencing: Total genomic DNA was extracted from the dried leaf tissue of the plant specimen with the help of a modified version of CTAB (Sunanta *et al*., 2020). The chloroplast barcode such as *matK*, *rbcL*, *trnH-psbA* and nuclear barcode ITS were used for amplification. The PCR was performed in a 25µl reaction mixture containing the master mix ingredients with the final concentration of 20 µM $MgCl_2$, 10 µM dNTPs, 0.01 µM of each primer, 0.25 U *Taq* (Biotaq^{TM} Polymerase, Bioline), and the template DNA was 1 µl. Along with theses reagents, Betain and BSA were also added to enhance the performance of PCR. To increase the rate of PCR success Betain and BSA were used. For each primer, PCR conditions and reagents are shown in Table 2. The conditions of *rbcL* and *trnH-psbA* were the same. For *rbcL* and *trnH-psbA*. After initial denaturation at 94° C, 35 cycles of 45s at 94° C, 30s at 53° C and 1 min at 72° C final extension were optimized. For ITS, an initial denaturation at 95° C 37 cycles of 20s at 95° C, 1 min at 55° C and 2 min at 72° C were performed. PCR products were checked on 1% agarose gel in TAE buffer. The PCR products were cleaned by using DNeasy Plant Mini Kit (Qiagen), or GFX PCR DNA and gel band purification kit IIlustra (GE Healthcare). The samples were then eluted in 20 µl of Nanopure clean water. The successful amplification products were submitted for Sanger sequencing at Biomedical Laboratories at the Natural History Museum, London, UK. The primer sequences are shown in Table 2.

Table 2. Primers sequences.

Name of Barcodes	Sequences (5′ to 3′)	References
ITS F	GGAAGTAAAAGTCGTAACAAGGTCCTCCGCTTATTGATATGC	White *et al.,* 1990
ITS R		
rbcL IF	ATGTCACCACAAACAGAGACTAAAGCGCAGCAGCTAGTTCCGG	Hasebe *et al*.,1994
rbcL 1351	GCTCCA	
trnH-psbA-F	GTTATGCATGAATGCTCCGCGCATGGTGGATTCACAATCC	Hamilton, 1990
psbA- trnH-R		

Sequence Analysis: The new sequences were assembled and edited by using Sequencher v.4.8 (Gene Codes Corporation and its licensors 1991-2007). The Geneious 6.0.5 to 7.1.7 was used to generate the consensus sequences from different contigs of forward and reverse sequences. All the successful sequences were submitted in the GenBank.

After editing, the sequences were aligned by using MAFFT aligner v6.240 for further analysis. BioEdit 7.2.1 was used to edit the aligned biological sequences and to trim the roughly arranged data. To select the highest similar sequences, the consensus sequences were run on the BLAST searches of the NCBI database. Genetic distance and nucleotide variation were examined based on Kimura 2 Parameter (K2P) model and Neighbour-Joining Tree (NJT) was constructed in MEGA 6.06 (Tamura *et al.,* 2013). The bootstrap value was 1000 replicates, with rates and patterns of the gamma distribution method. For the discriminatory species, success "Best Match'' (BM) and ''Best Close Match'' (BCM) tests in Species Identifier 1.8 program of TAXONDNA software (Meier *et al*., 2006) were used.

Inter- and intra-specific divergence: To estimate the level of divergence, variable sites, and informative sites among species, the pairwise p-distance was calculated in MEGA 6. The distributed frequency of inter- and intra-specific genetic divergence among all tested barcode regions was evaluated in TAXONDNA (Pang *et al*., 2011).

Similarity-based method: Numerous methods were used for the identification of species to assist the resolution power of the tested DNA barcode regions. For similarity-based assessment, TAXONDNA was used. In the 'best match' method, the identification was successful if all the sequences were from the same species. However, if the sequences were not from the same species, the success was regarded as incorrect. In some cases, several sequences showed good matches from different species, and was considered 'ambiguous'. The 'best close match' method was difficult when pairwise distances were calculated according to the Meier *et al*. (2006), so the threshold was set to 95%. If the results showed values above the threshold, the characters were treated as 'no match'. However, if the results showed values below the threshold, the characters were treated as 'best match'.

Tree-based method: In tree-based analysis, Neighbour-Joining (NJ) trees were constructed using MEGA 6. For the assessment of our datasets whether these sequences formed a specific species cluster or occurred in a separate clade, Kimura 2 parameter model was selected, and node support was 1000 bootstrap replicates. In Neighbour-joining trees, species forming a monophyletic clade with multiple individuals having the bootstrap value above 60% were said to be a successful identification. (Krawczyk *et al*. 2014).

BLAST score-based: In Mega BLAST algorithm, the BLASTClust thoroughly clustered all the DNA sequences on the bases of pairwise matches. BLAST search was performed for the sequences to combine all the matching sequences on the database. Usually, BLASTClust selects all the sequences that have statistically good matches with the query sequence and clusters all the identical sequences (Kress *et al*. 2010). To select the best match from the BLAST, sequences will need to be above 80% identical rate over query coverage of 100%. If the sequence has identical DNA sequences on the BLAST, then the identification is treated as a correct identification. However, if the sequence has DNA sequences from different species, then the result is considered a failure in identification.

RESULTS

The suitability of the universal barcode was investigated based on PCR amplification, sequence universality, and species delimitation. The characteristic of an ideal barcode region is that this region must have a conserved sequence. When it has such a sequence, it can easily be amplified, and have a high power of species discrimination.

Sequencing and PCR Success Rate: In the present study, four commonly used barcodes, were tested. All tested barcodes performed differently in term of universality in amplification and sequencing rates. In this study *matK* failed to amplify, *rbcL* came up with highest PCR amplification rate of 100%, *trnH-psbA* 92%, and ITS came up with 70% amplification rate. *rbcL* also showed good performance in sequencing with 100% success rate, followed by *trnH-psbA* with an 88.55%, with ITS an 80% success rate. Similarly, out of all tested barcodes, the Sequence accusation rate for *rbcL* was 67% followed by ITS with 45%. Among the tested barcodes *trnH-psbA* showed the lowest rate of success in the present study. The species identification according to the BLAST was high for *rbcL* with highly variable sites followed by ITS shown in (Table 3).

Phylogenetic Trees: Trees were constructed in MEGA 6.0 using the neighbour-joining (NJ) method based on the Kimura 2 parameter model. The node support was calculated at 1000 replicates. The result showed that the average node support for phylogenetic trees of three candidate barcodes *rbcL*, trnH-psbA, and ITS was 100%, 53% and 100% respectively. The phylogenetic tree emerged for *rbcL* with three clades, in which the present study species occur in II clade showed close relation with *Anemone canadensis* (JX848514.1). in the *trnH-psbA* the tree contained only one clade, the studied species showed close relation with *Anemone narssiflora* (MF543612.1) while the ITS tree showed two clades, in which the investigated species occur in II clade and showed close similarity with *Anemone taipaiensis* (MH711391.1) as shown in Fig. 1, 2 and 3.

Genetic Distances: The genetic pairwise distance between sequences showed the nucleotide divergence in the sequences. The largest and smallest values for tested barcodes are shown in bold in Table 4. The result showed that the largest distance was found in *Anemone demissa* while the smallest value was found in the *Anemone patens.* For ITS, the maximum distance was found in *Anemone obtusiloba* (LC511740) and the minimum distance was in *Anemone demissa* (KX 984124.1). The result of the distance matrix showed moderate differences between all sequences. Similarly, we also examined the result based on interspecific and intraspecific divergence in the three tested barcodes. All the barcode regions had very low intraspecific divergence. The intraspecific distances of the barcodes never exceeded 0.00. Moreover, ITS and *trnH-psbA* had slightly lower interspecific distance as compared to the *rbcL* shown in Table 4.

Species identification: The results on the bases of similarity implemented by TAXONDNA are shown in Table 5. In the result, the *rbcL* had the maximum rate of correct identification (80.55%), followed by ITS (70.41 %) and *trnH-psbA* (40.5 %). During BLASTClust analysis, *rbcL* showed a high-resolution rate of 67% followed by ITS 45%, and that had the high efficiency of identification supported by TAXONDNA on the basis of the correct identification method (Table 5).

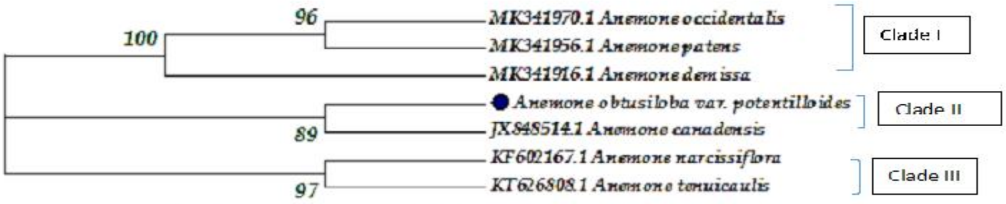


Fig. 1. Phylogenetic tree based on Neighbour joining tree of *rbcL*.

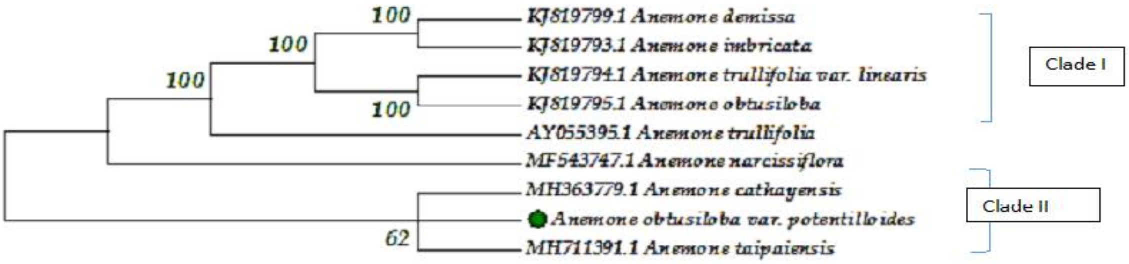


Fig. 2. Phylogenetic tree based on Neighbour joining tree of ITS.

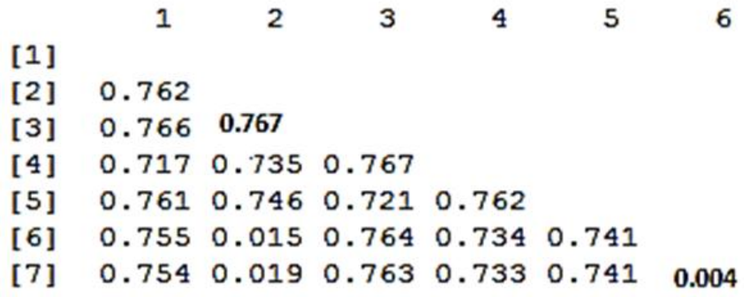
Fig. 3. Phylogenetic tree based on Neighbour joining tree of trnH-psbA.

Table 3: Summary of the variability of five DNA barcode regions.

BLAST	ITS	*trnH-psbA*	*rbcL*
Sequence length	617	397	1142
Trim length	523	299	1142
Total character	523	299	1142
Conserved site	0	0	01
Variable sites	523	299	1141
Informative sites	465	243	972
Identity	96%	93%	98%
Query coverage	100%	100%	100%
E.value	0.0	0.0	0.0
PCR Success	70%	92%	100%

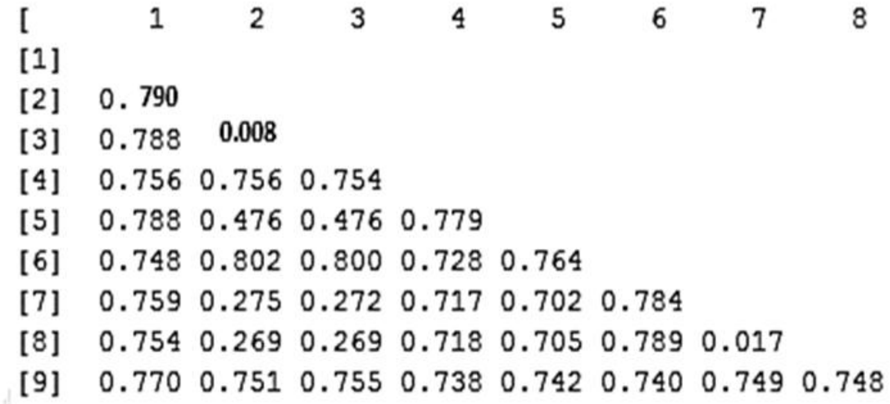
Sequence acquisition	45%	40%	65%
Intraspecific	0.00	0.00	0.00
Interspecific	0.750±0.750	0.720±760	0.764±765

Table 4. A. Evolutionary divergence between sequences of *rbcL*.
(1) *Anemone ranunculoides*, (2) *Anemone demissa* (3) *Anemone narssiflora* (4) *Anemone Canadensis* (5) *Anemone tenuicaulis* (6) *Anemone occidentalis* (7) *Anemone patens*.



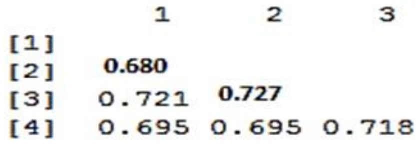
```
       1     2     3     4     5     6     7
[1]
[2]  0.762
[3]  0.766 0.767
[4]  0.717 0.735 0.767
[5]  0.761 0.746 0.721 0.762
[6]  0.755 0.015 0.764 0.734 0.741
[7]  0.754 0.019 0.763 0.733 0.741  0.004
```

Table 4. B. Divergence between sequences of ITS.
(1) *Anemone ranunculoides,* (2) *Anemone demissa*, (3) *Anemone imbricate,* (4) Anemone cathayensis, (5) *Anemone trullifolia,* (6) *Anemone taipaiensis,* (7) *Anemone trullifolia var linearis,* (8) *Anemone obtusiloba,* (9) *Anemone narssiflora.*



```
[      1     2     3     4     5     6     7     8     9
[1]
[2]  0. 790
[3]  0.788 0.008
[4]  0.756 0.756 0.754
[5]  0.788 0.476 0.476 0.779
[6]  0.748 0.802 0.800 0.728 0.764
[7]  0.759 0.275 0.272 0.717 0.702 0.784
[8]  0.754 0.269 0.269 0.718 0.705 0.789 0.017
[9]  0.770 0.751 0.755 0.738 0.742 0.740 0.749 0.748
```

Table 4. C. Divergence between sequences of *trnH-psbA*.
(1) *Anemone ranunculoides*, (2) *Anemone narcissiflora*, (3) *Anemone demissa*, (4) *Anemone Americana.*



```
       1     2     3     4
[1]
[2]  0.680
[3]  0.721 0.727
[4]  0.695 0.695 0.718
```

Table 5: Identification based on the success of the Best match and Best Closed Match and All Species Barcodes by TaxonDNA.

Best Match		Best Close Match			All Species Barcodes				
Gene	**Correct**	**Ambiguous**	**Incorrect**	**Correct**	**Ambiguous**	**Incorrect**	**Correct**	**Ambiguous**	**Incorrect**
ITS	70.41	9.20	8.6	70.29	0.0	5.2	82.20	7.2	4.3
trnH-psbA	40.5	0.0	20.65	28.5	9.89	10.70	50.31	40.71	1.80
Rbcl	80.55	0.0	25.70	82.55	0.0	5.65	57.41	11.18	7.8

DISCUSSION

Floral biodiversity, especially the diversity of medicinal plant species, is important because it is an income source for local people. By studying these species of plant scientists can also gather information about their medicinal uses as preserved in local traditions (Xiong and Long 2020). DNA Barcoding is recommended as an effective method for species identification, as it can identify outdated as well as new species (Xiong *et al*., 2018).

Initially, in plant DNA Barcoding, *rbcL* and *matK* were recommended as the universal barcode regions. While the *trnH-psbA* and ITS were used as supplementary barcodes (Chen *et al.*, 2020). In the present study, four candidate barcode loci where the efficacy of barcode regions was evaluated. Our study supports the effectiveness of DNA barcoding as a species identification tool, which in turn is necessary for the conservation of investigated species. This is the first study on the assessment of DNA barcode regions in *Anemone obtusiloba* from the Western Himalayan region of Pakistan. All studied barcodes showed reasonably good results except *matK* which showed worse performance in amplification as compared to other barcodes. In some previous literature, researchers described the PCR and sequencing problems with *matK* gene (Saha *et al*., 2020).

For the selection of a suitable barcode, the PCR and sequencing rate are the most important criteria (Fernandes *et al*., 2020). In this study, two universal barcodes *matK* and *rbcL,* and two supplementary barcodes *trnH-psbA* and ITS, were investigated to screen the suitable barcode for the identification of *Anemone obtusiloba* var. *potentilloides.* The performance of the DNA barcodes was tested based on primer universality, successful species identification rate and phylogenetic tree. In this regard, the *rbcL* had the best performance among all tested barcode regions (Wu *et al*., 2019).

The result of the present study is consistent with the previous literature that showed the success rate of *rbcL* to be 90% (Pei *et al*., 2017; Khan *et al*., 2019a; Wu *et al*., 2019). Although the *trnH-psbA* barcode region has high variation in a different group of plants. But the amplification and sequencing of *trnH-psbA* is difficult due to the presence of single nucleotide repeats and insertions and deletions. In some studies, the *trnH-psbA* revealed worst performance which is opposite to the present study in which *trnH-psbA* showed slightly good performance in sequencing, 92% (Tripathi *et al*. 2013). In some literature the success rate of sequencing for *trnH-psbA* was 100% (Chen *et al*., 2020). The amplification and sequencing of *matK* region has been remained the challenge and similarly we failed this region to amplify in our samples (Khan *et al*., 2019a). In various literature with comparison to the plastid genome the nrDNA mentioned as highly effective barcode region, and ITS has showed the highest rate of amplification as well as sequencing rate, ITS was recommended as an efficient barcode for the identification of some plant species (Gao *et al*. 2010; Dhivya *et al*., 2020; Sijimol *et al*., 2020). Although ITS has large variable sites compared to the plastid region the lowest success rate of species resolution and discrimination power (Chen *et al*., 2020)

In conclusion, we applied and screened suitable DNA barcodes for endemic plant species where *rbcL* had successfully been executed as a novel DNA barcode for correct identification of *Anemone obtusiloba* var. *potentilloides* endemic to Pakistan

Conclusion: Based on the sequencing, BlAST, TAXONDNA and phylogenetic analysis it is concluded that DNA barcoding is a quick, accurate and universal species identification method which can refine and conveniently discriminate relatively similar species. So, we suggest *rbcL* as a most suitable barcode marker for the identification of the *Anemone obtusiloba* var. *potentilloides*.

Conflict of interest: The authors declare no conflict of interest regarding the publication of this manuscript.

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Author contributions: SU designed the study, collected plant material, performed experiments, data analysis, and wrote the articles. KM provided main guidance during experimentation and helped in writing the main article. AM provided the plant material and proofread of article. AD had contributed to final draft corrections and provided technical assistance in improving the quality of the final draft. HA provided guidance for identification of plant and proofreading of the article

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