

# Evaluation of mat K and rbc L genes as markers in DNA barcoding of *Codiaeum variegatum* (L.)

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## 1 Evaluation of *matK* and *rbcl* genes as markers in DNA barcoding of *Codiaeum variegatum* (L.) Blume.

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*Codiaeum variegatum* (L.) Blume., commonly referred to garden croton, has variation in leaf shape and colour. The molecular identification using DNA barcode in this species has been scarcely carried out. 33 In this study we focused on two cultivars with different leaf shape and color, i.e. gold star and royal. The aim of this study was to evaluate *matK* and *rbcl* genes as markers in DNA barcoding of croton and to provide recommendation which marker was to be used in identifying croton more properly. The DNA extraction used innuPREP Plant DNA Kit and the Kit PCR 5x FirePol Master Mix was used to amplify *matK* and *rbcl* genes fragments using available universal primers. Sequence alignment using *matK* and *rbcl* showed that both varieties were 100% identical. Sequence identification of *matK* revealed 98.96% similarity with *Philodendron radiatum*, *Monstera* sp. and *Homalomena spariiae* using BOLD Systems and 100% similarity with *H. asperifolia* and *H. asmae* using BLAST. Sequence identification of *rbcl* gene using BOLD Systems and BLAST demonstrated that both varieties had 100% similarities with *C. variegatum*. In conclusion, *rbcl* gene was more reliable to be used as DNA barcode for identification of *C. variegatum* than *matK* gene.

Keywords: barcode, *Codiaeum variegatum*, *matK*, *rbcl*

### 7 INTRODUCTION

*Codiaeum* is the second largest genus of the family Euphorbiaceae and it is native plant in the Mollucan Islands of Indonesia 27, as well as Philippines, Thailand, Malaysia, New Guinea, Australia, India, Sri Lanka 11 and some other Pacific Islands. Garden croton (*Codiaeum variegatum* (L.) Blume.) is a group of small evergreen trees, perennial, tropical ornamental herbs and shrubs, with variation in leaf shape and colour. Leaf shapes vary from simple ovate to linear; some are slightly or deeply cut, and others are connected with the blade only by the midrib. The leaf color performed as shades, blends, combinations, or solid patches of red, pink, orange, yellow, lavender, black, and green (Deng et al., 2010). The variations of leaf color in crotons could be related to the combination of produced pigment in

the plants, such as chlorophyll, carotene, 30 phaeophytin, xanthophyll (Ogunwenmo et al. 2007) and anthocyanin (Papafotiou et al., 2007). 11 addition to its ornamental value, croton phytochemicals are known to have protective and preventive properties against diseases as well as antioxidants (Ogunwenmo et al., 2007; Deng et al., 2010).

Reliable methods to identify and distinguish ornamental plants specimen may help in solving the problem of doubt caused by counterfeited ornamental plants and many other 6 illegal activities in the horticultural industry. Traditionally, the identification and characterization of cultivars and species was based on morphological and physiological properties, however, this identification is not effective and reliable (Elansary et al., 2017). In recent days the identification

method has been developed using molecular markers, such as DNA barcode (Hebert et al. 2003; Kadkhodaei et al., 2010) to analyze the diversity of plants and to determine plant specimens to their species although the morphological diagnostic characters are unavailable (Elansary et al., 2017).

As a technique for taxonomic identification, DNA barcoding can utilize one or several standardized DNA regions that are universally present in the target lineages and have sufficient sequence variation to recognize species and identify individuals correctly. DNA barcoding is a potential tool to detect error in identifying species because similarity-based approaches using DNA barcoding combined with morphology would solve the misidentification based on morphology (Liu et al. 2017). For the identification of plant species, two defined regions of the chloroplast DNA (maturase K or *matK* and ribulose-1,5-biphosphate carboxylase oxygenase large subunit or *rbcl*) have been widely used for standard barcodes as endorsed by the Plant Working Group of the Consortium for the Barcode of Life (CBOL) in 2009 (CBOL, 2009). DNA barcode served fast and accurate identification of a plant species, and the sequences are available in the sequence library such as GenBank and BOLD (Kress and Erickson, 2007). Ogunwenmo et al., (2007) reported that cultivars of *C. variegatum* showed variability in content of pigments and chromosome numbers. Deng et al., (2010) studied genetic relationship of 44 cultivars of *C. variegatum* using amplified fragment length polymorphism (AFLP) markers. The molecular identification using DNA barcode in croton, however, has been scarcely carried out. There are so many cultivars of croton, but in this study we focused on two cultivars with different leaf shape and color, i.e. gold star and royal. The study aimed to evaluate *matK* and *rbcl* genes as markers in DNA barcoding of croton and to provide recommendation which marker was to be used in identifying croton more properly.

## MATERIALS AND METHODS

### DNA Extraction, Amplification and Sequencing

Total DNA was extracted from approximately 50 mg of plant materials (gold star and royal) using innuPREP Plant DNA Kit (Analytik Jena, Germany) according to manual, with a slight modification to increase the chloroplast DNA yield (Kolondam, 2015). Genes of *matK* and *rbcl* were amplified using 5x Firepol PCR Master Mix Ready-to-load (Solis Biodyne). Primer pairs used

for gene *matK* were MatK-1RKIM-f 5'-ACCAG TCC ATT CTG GAA ATC TTG GTT C-3' and MatK-3FKIM-r 5'-CGT ACA GTA CTT TTG TGT TTA CGA G-3' (Kuzmina et al., 2012). Primer pairs used for gene *rbcl* were *rbcl*LaF 5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3' and *rbcl*LaR 5'-GTA AAA TCA AGT CCA CCR CG-3' (Kress and Erickson, 2007). Amplification was performed as follows: predenaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and polymerization at 72°C for 50 sec. Amplicons were separated using 1% agarose gel. Clear cut bands indicated the success of the amplification. The PCR products together with their primer pairs were sent to 1Base Malaysia for sequencing.

### Data Analysis

The chromatograms were corrected using Geneious v5.6 (Kearse et al., 2012), and then processed using other available online programs, as suggested by Tallei and Kolondam (2015). The sequences were pairwise aligned using global alignment with free ind gaps to identify regions of 95% similarity. Consensus sequences were generated by pairwise alignment of forward and reverse sequences using MUSCLE (Multiple Sequence Comparison by Log-Expectation) which is integrated in Geneious v5.6.

The sequences generated using each marker were aligned respectively, using multiple sequence alignment (multalin) with hierarchical clustering (Corpet, 1988; <http://multalin.toulouse.inra.fr/multalin>), and trimmed accordingly to get the core area of *matK* and *rbcl*. The croton plants were identified using BOLD (Barcode of Life Database) Systems ([www.boldsystems.org](http://www.boldsystems.org)) (Ratnasingham and Hebert, 2007). The identification was correct if the highest identity percentage of searched sequences was derived from expected species or genus. On the other hand, the identification was ambiguous when the highest identity percentage of searched sequences was not derived from expected species or genus, or family. A homology search for *matK* and *rbcl* genes was performed using Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### RESULTS AND DISCUSSION

Sequence alignment of *matK* gene using multalin showed that *Codiaeum variegatum* cv. Gold star (SPG2) and Royal (JM2) were 100% identical (Figure 1).

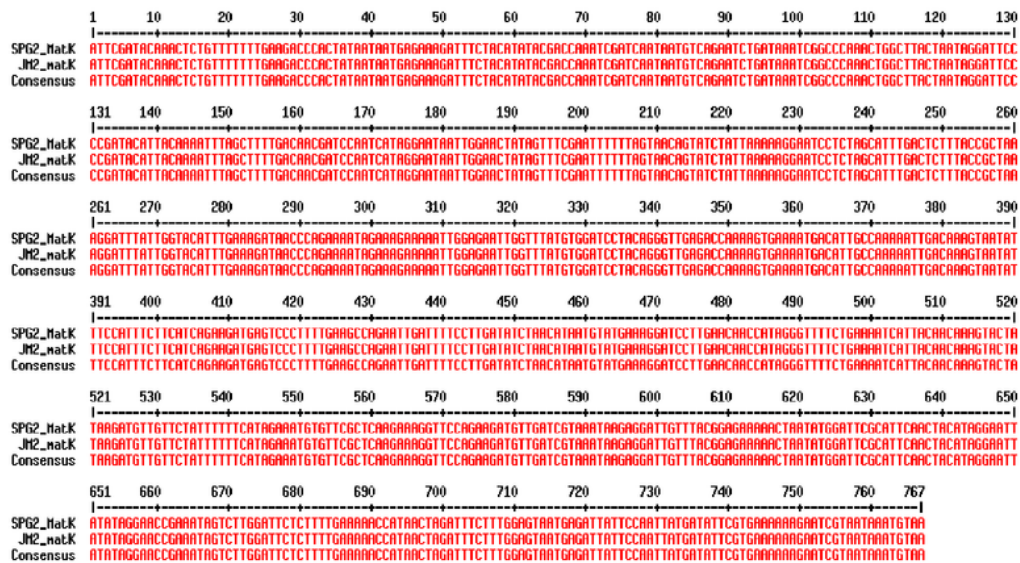


Figure 1. Sequence alignment of *matK* gene of *Codiaeum variegatum* cv. Gold star (SPG2) and royal (JM2) using Multalin (<http://multalin.toulouse.inra.fr/multalin/>).

| Match Rank | Phylum        | Class      | Order       | Family  | Genus               | Species                | Subspecies | Score | Similarity | E-Value | Status        |
|------------|---------------|------------|-------------|---------|---------------------|------------------------|------------|-------|------------|---------|---------------|
| 1          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Philodendron</i> | <i>radiatum</i>        |            | 751   | 98.96      | 0       | Early-Release |
| 2          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Monstera</i>     |                        |            | 751   | 98.96      | 0       | Early-Release |
| 3          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Monstera</i>     |                        |            | 751   | 98.96      | 0       | Early-Release |
| 4          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Homalomena</i>   | <i>speariae</i>        |            | 750   | 98.96      | 0       | Published     |
| 5          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Philodendron</i> | <i>fragrantissimum</i> |            | 749   | 98.83      | 0       | Published     |
| 6          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Philodendron</i> | <i>fragrantissimum</i> |            | 749   | 98.83      | 0       | Published     |
| 7          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Philodendron</i> | <i>sulcatum</i>        |            | 747   | 98.7       | 0       | Early-Release |
| 8          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Philodendron</i> | <i>jacquini</i>        |            | 745   | 98.57      | 0       | Early-Release |
| 9          | Magnoliophyta | Liliopsida | Alismatales | Araceae | <i>Furtdoa</i>      | <i>mixta</i>           |            | 744   | 99.73      | 0       | Published     |
| 10         | Magnoliophyta | Liliopsida | Alismatales | Araceae |                     | <i>Jorge170</i>        |            | 741   | 99.08      | 0       | Published     |

Figure 2. Identification based on BOLD System of *matK* gene of *Codiaeum variegatum* cv. Gold star (SPG2) and royal (JM2).

Although both varieties have different characteristics in leaf shape and color, this result revealed that they are the same species. The similar results were reported in green daluga, yellow daluga and mottled daluga from Sangihe Islands which included in the same species, i.e. *Cyrtosperma merkusii* (Julianti et al., 2015) and ornamental plant *Sansevieria trifasciata* var. Laurentii and Hahnii (Tallei et al., 2016a). Partial fragment of *matK* gene amplified using primer pairs of MatK-1RKIM and MatK-3FKIM-r did not indicate intraspecific variation between these

croton cultivars.

Sequence identification of *matK* gene using BOLD Systems (Figure 2) revealed 98.96% similarity with *Philodendron radiatum*, *Monstera* sp. and *Homalomena speariae*. The same sequence of *matK* gene employed in BLAST demonstrated 100% similarity with *Homalomena asperifolia* and *Homalomena asmae* (Figure 3). The similar result was demonstrated by Tallei and Kolondam (2015) which showed that using *matK* gene, Sangihe nutmeg (*Myristica fragrans*) had 100% similarity with *M. fatua*, *M. maingayi*, and *M. globosa*.

**Descriptions**  
Sequences producing significant alignments:

| Description  | Max score | Total score | Query cover | E value | Ident | Accession                  |
|--|-----------|-------------|-------------|---------|-------|----------------------------|
| Homalomena asperifolia voucher Kelsuke Hase Ar4761 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast               | 1417      | 1417        | 100%        | 0.0     | 100%  | <a href="#">KM580706.1</a> |
| Homalomena sp. Kelsuke Hase Ar4759 voucher Kelsuke Hase Ar4759 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast   | 1417      | 1417        | 100%        | 0.0     | 100%  | <a href="#">KM580705.1</a> |
| Homalomena sp. Baharuddin Ar2597 voucher P.C.Boyce et al. Ar3047 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast | 1417      | 1417        | 100%        | 0.0     | 100%  | <a href="#">KM580692.1</a> |
| Homalomena asmae voucher Baharuddin Ar2597 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast                       | 1417      | 1417        | 100%        | 0.0     | 100%  | <a href="#">KM580691.1</a> |
| Homalomena asmae voucher AR2597 rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, partial cds; chloroplast   | 1417      | 1417        | 100%        | 0.0     | 100%  | <a href="#">JX024970.1</a> |
| Homalomena sp. Melaka rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, partial cds; chloroplast   | 1417      | 1417        | 100%        | 0.0     | 100%  | <a href="#">JX024968.1</a> |
| Homalomena tonkinensis voucher P.C.Boyce et al. Ar4302 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast           | 1411      | 1411        | 100%        | 0.0     | 99%   | <a href="#">KM580707.1</a> |
| Homalomena curvata voucher P.C.Boyce et al. Ar3052 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast               | 1411      | 1411        | 100%        | 0.0     | 99%   | <a href="#">KM580702.1</a> |
| Homalomena atrox voucher P.C.Boyce et al. Ar2389 (SAR) rRNA-Lys (trnK) gene, partial sequence; and maturase K (matK) gene, complete cds; chloroplast                 | 1411      | 1411        | 100%        | 0.0     | 99%   | <a href="#">KM580701.1</a> |

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Figure 3. Identification based on BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of matK gene of *Codiaeum variegatum* cv. Gold star (SPG2) and royal (JM2).



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Figure 4. Sequence alignment of rbcL gene of *Codiaeum variegatum* cv. Gold star (SPG2) and royal (JM2) (<http://multalin.toulouse.inra.fr/multalin>).

| Match Rank | Phylum        | Class         | Order        | Family        | Genus                 | Species             | Subspecies | Score | Similarity | E-Value | Status                      |
|------------|---------------|---------------|--------------|---------------|-----------------------|---------------------|------------|-------|------------|---------|-----------------------------|
| 1          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Codiaeum</i>       | <i>variegatum</i>   |            | 622   | 100        | 0       | Published <a href="#">↗</a> |
| 2          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Codiaeum</i>       | <i>peltatum</i>     |            | 620   | 99.84      | 0       | Published <a href="#">↗</a> |
| 3          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Strophoblachia</i> | <i>fimbriicalyx</i> |            | 612   | 99.2       | 0       | Published <a href="#">↗</a> |
| 4          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Blachia</i>        | <i>siamensis</i>    |            | 612   | 99.2       | 0       | Published <a href="#">↗</a> |
| 5          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Strophoblachia</i> | <i>fimbriicalyx</i> |            | 612   | 99.2       | 0       | Published <a href="#">↗</a> |
| 6          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Vernicia</i>       | <i>montana</i>      |            | 604   | 98.55      | 0       | Published <a href="#">↗</a> |
| 7          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Hylandia</i>       | <i>dockrillii</i>   |            | 604   | 98.55      | 0       | Published <a href="#">↗</a> |
| 8          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Ostodes</i>        | <i>paniculata</i>   |            | 604   | 98.55      | 0       | Published <a href="#">↗</a> |
| 9          | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Ostodes</i>        | <i>paniculata</i>   |            | 604   | 98.55      | 0       | Published <a href="#">↗</a> |
| 10         | Magnoliophyta | Magnoliopsida | Malpighiales | Euphorbiaceae | <i>Vernicia</i>       | <i>montana</i>      |            | 604   | 98.55      | 0       | Published <a href="#">↗</a> |

Figure 5. Identification based on BOLD System of *rbcl* gene of *Codiaeum variegatum* cv. Gold star (SPG2) and royal (JM2).

| Descriptions   |           |             |             |         |       |            |
|--|-----------|-------------|-------------|---------|-------|------------|
| Sequences producing significant alignments:  |           |             |             |         |       |            |
| Description  | Max score | Total score | Query cover | E value | Ident | Accession  |
| <i>Codiaeum variegatum</i> ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ( <i>rbcl</i> ) gene, partial cds; chloroplast              | 1149      | 1149        | 100%        | 0.0     | 100%  | AY788169.1 |
| <i>Codiaeum peltatum</i> chloroplast <i>rbcl</i> gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial cds                 | 1144      | 1144        | 100%        | 0.0     | 99%   | AB233876.1 |
| <i>Strophoblachia fimbriicalyx</i> ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ( <i>rbcl</i> ) gene, partial cds; chloroplast      | 1122      | 1122        | 100%        | 0.0     | 99%   | AY794901.1 |
| <i>Blachia siamensis</i> ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ( <i>rbcl</i> ) gene, partial cds; chloroplast                | 1122      | 1122        | 100%        | 0.0     | 99%   | AY794989.1 |
| <i>Strophoblachia fimbriicalyx</i> plastid partial <i>rbcl</i> gene for rubisco large subunit  | 1122      | 1122        | 100%        | 0.0     | 99%   | AJ418306.1 |
| <i>Vernicia fordii</i> chloroplast, complete genome  | 1105      | 1105        | 100%        | 0.0     | 99%   | KY628429.1 |
| <i>Vernicia fordii</i> isolate 01 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ( <i>rbcl</i> ) gene, partial cds; plastid           | 1105      | 1105        | 100%        | 0.0     | 99%   | KF822509.1 |
| <i>Vernicia fordii</i> voucher CPG09794 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ( <i>rbcl</i> ) gene, partial cds; chloroplast | 1099      | 1099        | 100%        | 0.0     | 99%   | KX527167.1 |
| <i>Vernicia montana</i> chloroplast <i>rbcl</i> gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial cds                  | 1099      | 1099        | 100%        | 0.0     | 99%   | AB267953.1 |

Figure 6. Identification based on BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of *rbcl* gene of *Codiaeum variegatum* cv. Gold star (SPG2) and royal (JM2).

Sequence alignment using *rbcl* gene showed that both varieties were 100% identical (Figure 4). Based on the identification of *rbcl* barcode using BOLD System (Figure 5) and BLAST, these two croton varieties had 100% similarities with *Codiaeum variegatum* (Figure 6).

The results of this study indicated that *rbcl* gene was more potential than *matK* gene as DNA barcode for identifying *Codiaeum variegatum* because these two croton varieties had 100% similarities with *Codiaeum variegatum* based on the identification of *rbcl* barcode using BOLD

System and BLAST. It was reported that ITS (internal transcribed spacer) and ITS 2 (internal transcribed spacer 2) were more potential than *rbcl* and *matK* as barcodes for identifying the Euphorbiaceae species. At the interspecific level, the highest divergence was provided by ITS 2 followed by ITS. The marker *rbcl* indicated the intraspecific divergence and there were no significant differences of divergences among *matK*, ITS 1 and ITS 2 (Pang et al., 2010). In addition, the combination of ITS+*matK* was recommended as the optimal DNA barcode for large flowering plant genera based on their

25 evaluation of DNA barcodes in *Dendrobium* (Orchidaceae) fr<sup>23</sup> Mainland Asia (Xu et al., 2015) as well as in *Codonopsis* (Campanulaceae) and in some large angiosperm plant genera (Wang et al., 2017). Mishra et al., (2017) also showed that multilocus regions had a higher discriminatory power than single barcodes, and the combination of matK+ITS showed the<sup>16</sup> highest resolution rate (94.44%). Furthermore, the most efficient DNA barcode for identifying species and genera was IT<sup>16</sup> in Apocynaceae (Selvaraj et al., 2015) and ITS in Lauraceae from China (Liu et al., 2017). Tallei et al., (2016b) suggested that DNA barcodes must produce a significant barcoding gap between interspecific divergence and intraspecific distance. Elansary et al., (2017) reported that although the core DNA barcodes cannot always discriminate species, but at least it is potential to control the market place of horticultural crops and protect copyrights of new species and cultivars.

The size and completeness of barcode databases affected the success rate of DNA barcode to differentiate closely related species such as reported in the identification of African rainforest trees (Parmentier et al. 2017). Several combinations of two or three barcodes have been proposed as core barcodes to increase the species identification success rate (Xu et al. 2015). It was generally considered that combining DNA barcodes could improve species identification. For example, the discrimination rates of the combinations var<sup>28</sup> from 10.6% to 32.6% with  $rbcl+matK < rbcl+matK+trnH-psbA < rbcl+matK+trnH-psbA+ITS2 < rbcl+matK+trnH-psbA+ITS$  at the species level in Lauraceae from China. The rate of sequence recovery as well as the discrimination power of DNA barcodes should be considered to use them as markers (Liu et al. 2017). It will be valuable to evaluate ITS, ITS 2, *trnH-psbA* as single barcode or combination of these DNA barcode with *rbcl* and *matK* to obtain higher identification success rate in some varieties of *Codiaeum variegatum*.

#### CONCLUSION

*rbcl* gene was more reliable to be used as DNA barcode for identification of *C. variegatum* than *matK* gene.

#### CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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#### AUTHOR CONTRIBUTIONS

SAN collected the plant samples and also wrote the manuscript. BJK carried out<sup>20</sup> the laboratory work and TET analyzed the data and also reviewed the manuscript. All authors read and approved the final version.

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