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FIRST RECORD OF THE FIVE DNA BARCODES OF THE NOTHOSPECIES COCOR BEBEK (*KALANCHOE* × *LAETIVIRENS*)

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SUMMARY

Cocor bebek (*Kalanchoe* × *laetivirens*) is a nothospecies. Specifically, it is a hybrid species that resulted from hybridization between two different species, namely, *Kalanchoe daigremontiana* and *Kalanchoe laxiflora*. The DNA sequences of this species are not yet available in GenBank but are important for its rapid identification. Therefore, this study aimed to isolate and analyze five DNA barcode sequences, i.e., *26S rRNA*, *internal transcribed spacer* (*ITS), trnL-trnL-trnF intergenic spacer* (IGS)*, ndhC-trnV IGS*, and *rpl16 intron*, of the nothospecies *Kalanchoe* × *laetivirens*. Plant samples were collected from Pekanbaru City, Riau Province, Indonesia. The DNA sequences of *26S rRNA*, *ITS, trnL-trnL-trnF IGS, ndhC-trnV IGS*, and *rpl16 intron* were obtained with lengths of 898, 684, 908, 954, and 937 bp, respectively, and registered in the GenBank database with the registration numbers MW286359, MW286360, MW286361, MW286362, and MW286363, respectively. BLASTn analysis on four DNA barcodes, except for *ITS*, showed that *Kalanchoe* × *laetivirens* had the highest similarity to *K. daigremontiana* with an identity value of 97.25%–99.90%, query cover of 100%, and E value of 0.0. The DNA barcodes *26S rRNA*, *ITS*, *trnL-trnL-trnF IGS*, *ndhC-trnV IGS,* and *rpl16 intron* obtained in this study revealed high variations among the studied accessions due to the presence of substitution and deleted mutations. A number of nucleotides critical for the molecular identification of this plant were generated in all of the five DNA sequences. The *trnL-trnF IGS* sequence produced eight critical nucleotides, and *26S rRNA* and *ndhC-trnV IGS* each produced one. On the basis of the nucleotide similarity between the two species, the results proved that *Kalanchoe* × *laetivirens* descended from *K. daigremontiana*.

Keywords: 26S rRNA, DNA barcode, *internal transcribed spacer, Kalanchoe daigremontiana*, *Kalanchoe* × *laetivirens, ndhC-trnV intergenic spacer*, *rpl16 intron*, *trnL-trnL-trnF intergenic spacer*

Key findings: Five DNA sequences were reported for the first time for the species *Kalanchoe* × *laetivirens*. These DNA sequences are indispensable for the molecular identification of this plant.

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INTRODUCTION

DNA barcoding is a molecular technique for the identification of an organism. In this technique, DNA barcodes, which are short DNA sequences with known locations in the organism's genome, are used. This technique is especially helpful to scientists who are not taxonomists and can be easily used to identify an organism (Hebert *et al*., 2003). Therefore, a number of DNA barcodes in the database that can be accessed by various researchers is necessary (Will and Rubinoff, 2004; DeSalle, 2006; Roslim *et al*., 2016).

Five DNA barcodes, among which two are from the nuclear genome (*26S ribosomal RNA* [*26S rRNA*] and *internal transcribed spacer* [*ITS*]), and three others are from the chloroplast genome (*trnL-trnL-trnF intergenic spacer* [*IGS*], *ndhC-trnV IGS*, and *rpl16 intron*), are commonly used in plant DNA barcoding (Biffin *et al*., 2006; Shaw *et al*., 2007; de-Groot *et al*., 2011; Bolson *et al*., 2015; Buerki *et al*., 2016; Roslim, 2017). Noncoding regions have higher mutation rates and variations than coding regions and can thus clearly differentiate cryptic species (Kress *et al*., 2005).

Ribosomes are composites of small and large subunits that are present in eukaryotic cells. The small subunit consists of one rRNA, namely,18S rRNA, and 35 ribosomal proteins. The large subunit comprises three kinds of rRNA, specifically, 26S/28S rRNA, 5.8S rRNA and 5S rRNA, and 50 ribosomal proteins. The DNA sequences encoding 18S rRNA, 5.8S RNA, and 26S/28S rRNA are arranged in one transcript. However, the DNA sequences encoding 18S rRNA and 5.8S rRNA were separated by ITS1. Meanwhile, 5.8S rRNA and 26S/28S rRNA are separated by ITS2. In plants, the length of the DNA sequence from ITS1– 5.8S rRNA–ITS2 is approximately 600 bp. The DNA sequence encoding 26S/28S rRNA in plant cells has a length of approximately 3.3 kbp (Gillespie *et al*., 2006; Porter and Golding, 2012; Sáez-Vásquez and Delseny, 2019).

trnL-trnF IGS is a spacer between the tRNA-Leucine and tRNA-Phenylalanine genes. In *Nicotiana tabaccum*, *Oryza sativa*, and *Marchantia*, tRNA-Leucine genes have three regions, namely, the trnL (UAA)'exon, intron, and trnL(UAA)3'exon, with the total lengths of 577, 614, and 389 bp, respectively. Meanwhile, the tRNA-Phenylalanine gene does not have introns. Furthermore, the spacers between the trnL (UAA)3'exon and trnF has lengths of 438, 324, and 158 bp (Taberlet *et al*., 1991). The average length of the tRNA-Leucine gene in plants is 362 bp (Shaw *et al*., 2007). DNA barcoding analysis using *trnL-trnF IGS* frequently involves the intron in trnL (UAA) and its spacer; this sequence is called *trnL- trnL-trnF IGS* (Taberlet *et al*., 1991; Shaw *et al*., 2007).

Similar to *trnL-trnF IGS*, in plants, *ndhC-trnV IGS* also possesses a 318–1800 bp spacer region between NADH-plastoquinone oxidoreductase subunit 3 and tRNA-Valine genes with an average length of 1146 bp. *ndhC-trnV IGS* is a new plant DNA barcode with high variation; the variation shown by *ndhC-trnV IGS* is even higher than that shown by *trnL-trnF IGS* and *rpl16 intron*. *rpl16 intron* is a noncoding region of the gene encoding ribosomal protein 16 with a large subunit. Its average intron length noted in some plants is approximately1031 bp (Shaw *et al*., 2007).

Cocor bebek is a member of the Crassulaceae family and the Kalanchoideae subfamily. Genus *Kalanchoe* is divided into three subgenera, namely, *Bryophyllum*, *Kalanchoe*, and *Kitchingia*, on the basis of morphological, anatomical, embryological, karyological, and phytogeographical information and molecular genetics (Chernetskyy, 2011).

Kalanchoe has 150 described species (Descoings, 2006). *Kalanchoe* itself refers to plantlets that grow on the margins of the leaves of many *Kalanchoe* species and that transform into new plants if they fall on the ground (Descoings, 2003).

Many crosses that have occurred naturally and artificially have enriched the ornamental characteristics of *Kalanchoe* species. Crosses can produce fertile and sterile hybrids because that are easy to grow and to undergo transition into vegetative and reproductive phases via leaf adventitious shoots; thereofore, large morphological variations exist among the species of this genus (Shaw, 2008).

Some species of cocor bebek are members of the *Kalanchoe* genus, i.e., *Kalanchoe delagoensis*, *Kalanchoe rosei* var. *variifolia*, *Kalanchoe daigremontiana*, *Kalanchoe* × *houghtonii*, and *Kalanchoe* × *laetivirens* (Shaw, 2008). *Kalanchoe* × *laetivirens* was originally thought to be a native of Madagascar. However, recent findings have revealed that this plant is a nothospecies and a hybrid of *K. daigremontiana* and *Kalanchoe laxiflora* (syn. *Bryophyllum crenatum*) (Smith, 2020). The DNA barcode data for this plant are not yet available in the GenBank database. DNA barcodes are needed to identify these plants rapidly without having to wait for the plants to bloom (Kress *et al*., 2005; Rubinoff *et al*., 2006). Therefore, this study aimed to analyze the usefulness of five DNA barcodes in *Kalanchoe* × *laetivirens* for molecular identification.

MATERIALS AND METHODS

Genetic material and procedure

Kalanchoe × *laetivirens* plant samples were procured from the surroundings of Pekanbaru City, Riau Province, Indonesia. The primer pairs for amplifying the five DNA barcodes are presented in Table 1. Morphological observations were carried out on stems and leaves before the blooming and flowering stages in reference to Shaw (2008) and Smith *et al*. (2019). The characters observed were plant height; stem surface; internode length; phyllotaxis; petiole color and length; and leaf color, shape, length, and width.

Leaves were taken from the second and third orders of shoots for DNA isolation. Leaf samples were collected by using sterilized scissors and then labelled. The leaf samples were cleaved, and the mucus inside the leaves was removed. Next, 0.1 g of the leaf sample was cut off for total DNA extraction.

Total DNA extraction was performed by using Genomic DNA Mini Kit Plant (Geneaid). A sufficient amount of liquid nitrogen was poured into a mortar. Then, the leaves were ground until powdered. The powder was then transferred into a 1.5 ml tube, and DNA extraction was performed by following the manufacturer's instructions (Geneaid).

Table 1. Primers for the amplification of the five DNA barcodes.

Primers	5'----------3'	Ta (°C)	Regions	Reference
26S_NL1_F 26S_LR5_R	GCATATCAATAAGCGGAGGAAAAG ATCCTGAGGGAAACTTC	56.1	*26S ribosomal RNA*	Porter and Golding (2012)
FP_ITS5_F FP_ITS4_R	GAAAGTAAAAGTCGTAACAAGG TCCTCC GCTTATTGATATGC	47.0	*internal transcribed spacer*	Schoch *et al.* (2012); Wang *et al*. (2014)
B49317_F2 A49855_R2	CGAAATCGGTAGACGCTACG GGGGATAGAGGGACTTGAAC	51.8	*trnL(UAA)*	Taberlet *et al*. (1991)
B49873_F3 A50272_R3	GGTTCAAGTCCCTCTATCCC ATTTGAACTGGTGACACGAG	50.6	*trnL(UAA) 3'exon-trnF* intergenic spacer	
NdhC trnV$^{(UAC)}$x2	TATTATTAGAAATGYCCARAAAATATCATATTC GTCTACGGTTCGARTCCGTA	48.7	*ndhC-trnV* intergenic spacer	Shaw *et al.* (2007)
rpl16_F71 rpl16_R1516	GCTATGCTTAGTGTGTGACTCGTT CCCTTCATTCTTCCTCTATGTTG	47.0	*rpl16 intron*	Li *et al.* (2010)

The total DNA obtained was then checked for quality and quatity by using electrophoresis technique. Subsequently, the total DNA was stored at 4 °C for the following processes.

DNA targets were amplified via polymerase chain reaction (PCR) technique by using components and programs in reference to Roslim (2017). The thick and correct bands of the amplicon (40 µl per sample) and the forward (30 µl per sample) and reverse (30 µl per sample) primers were then sent to PT Genetika Science in Jakarta as an intermediete agent for sequencing in First Base Malaysia.

Data analysis

The DNA sequences that were obtained by sequencing with the forward and reverse primers were then aligned to obtain the correct sequences by using BioEdit version 7.0.0 software (Hall, 1999). The DNA sequences were then aligned with BLASTn program at http://www.ncbi.nlm.nih.gov/BLAST (Altschul *et al*., 1997) for the analysis of similarity with the DNA sequences that had been deposited in GenBank database. The sequences of the top of 10 species that appeared in BLASTn analysis were selected for the subsequent analysis, such as the determination of nucleotide differences and the construction of dendrograms, by using MEGA version 6.06 software (Tamura *et al*., 2013).

RESULTS

Morphological characteristics of *Kalanchoe* × *laetivirens*

Morphological characterization showed that *Kalanchoe* × *laetivirens* had a height of 1 m, smooth stem surfaces, internodes that were 2 cm to 5 cm in length, and young stems with smooth surfaces (Figure 1). The phyllotaxis was opposite dessucate, and the petiole was green and up to 8 cm in length. The leaves were green and glossy, oval, up to 20 cm in length and 7.5 cm in width and 0.1 cm to 0.2 cm in thickness. The leaves had slightly rough upper surfaces and smooth lower surfaces. They were rounded at the base with an obtuse tip. The margins were dentate and surrounded by small plantlets.

Total DNA and PCR products

The obtained DNA was amplified by using six primer pairs (Table 1). The obtained PCR products were thick and sufficient for sequencing. The DNA bands for *26S rRNA*, *ITS, trnL(UAA), trnL(UAA)3'exon-trnF IGS*, *ndhC-trnV IGS*, and *rpl16 intron* were approximately 900, 700, 550, 350, 900, and 1000 bp, respectively (Figure 2). The sizes of the DNA sequences were noted as 898 bp for *26S rRNA*, 684 bp for *ITS*, 908 bp for *trnL-trnL-trnF IGS* (comprising the 567 bp *trnL[UAA]* and the 353 bp *trnL[UAA]3'exon-trnF IGS*), 954 bp for *ndhC-trnV IGS*, and 937 bp for the *rpl16 intron*. These DNA sequences have been registered in GenBank database with the accession numbers MW286359, MW286360, MW286361, MW286362, and MW286363.

DNA sequence analysis of *26S rRNA*

The BLASTn analysis of *26S rRNA* sequences showed that *Kalanchoe* × *laetivirens* had the highest similarity to *K. daigremontiana* with the identity value of 99.89%, query cover of 100%, and E-value of 0.0. The lowest similarity was found with *Phyteuma spicatum* with the identity value of 95.66%, query cover of 100%, and E-value of 0.0 (Table 2).

One critical nucleotide that differentiated *Kalanchoe* × *laetivirens* from the other studied accessions was found at nucleotide number 22, namely guanine in *Kalanchoe* × *laetivirens* and adenine in the other studied accessions (Table 3). Meanwhile, four additional nucleotides could be used to distinguish *Kalanchoe* × *laetivirens* and *K. daigremontiana* from other studied accessions: nucleotides 86, 89, 94, and 204. At these positions, the nucleotides in

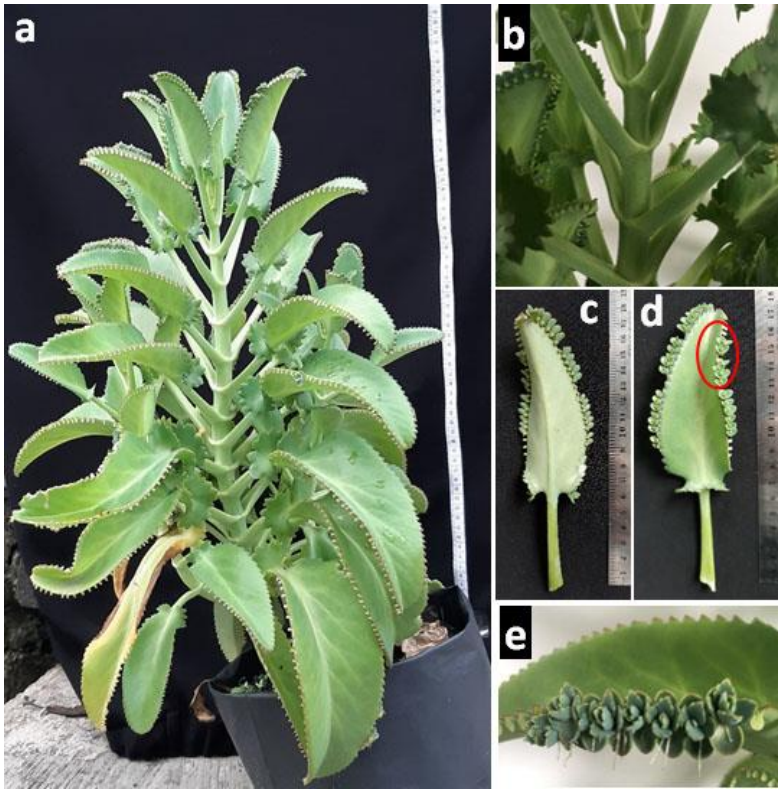

Figure 1. Morphological characteristics of *Kalanchoe* × *laetivirens*. (a) Plant habit, (b) stem exhibiting opposite dessucate phylotaxis, (c) lower leaf surface, (d) upper leaf surface (the circle indicates plantlets), and (e) magnified view of plantlets.

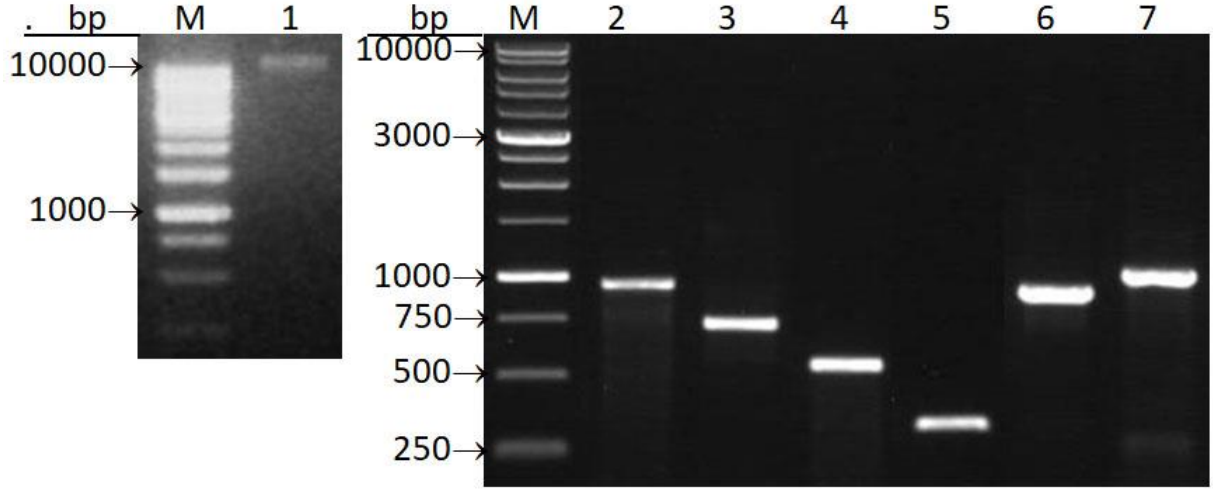

Figure 2. DNA bands of (1) total DNA, (2) *26S rRNA*, (3) *ITS*, (4) *trnL(UAA)*, (5) *trnL(UAA)3'exon-trnF intergenic spacer*, (6) *ndhC-trnV intergenic spacer*, and (7) *rpl16* intron of *Kalanchoe* × *laetivirens* separated on 1% agarose gel. (M) 1 kb DNA ladder (Thermo Scientific).

Table 2. BLASTn alignment analysis of the *26S rRNA* sequence of *Kalanchoe* × *laetivirens.*

No.	Species	Family	Query cover (%)	E-value	Identity (%)
1	*Kalanchoe daigremontiana*	Crassulaceae	100	0.0	99.89
2	*Dudleya viscida*	Crassulaceae	100	0.0	97.66
3	*Sedum nudum*	Crassulaceae	100	0.0	96.33
4	*Fendlera rupicola*	Hydrangeaceae	100	0.0	96.11
5	*Exbucklandia populnea*	Hamamelidaceae	100	0.0	96.00
6	*Mytilaria laosensis*	Hamamelidaceae	100	0.0	95.77
7	*Cornus officinalis*	Cornaceae	100	0.0	95.77
8	*Disanthus cercidifolius*	Hamamelidaceae	100	0.0	95.77
9	*Cercidiphyllum japonicum*	Carcidiphyllaceae	100	0.0	95.66
10	*Phyteuma spicatum*	Campanulaceae	100	0.0	95.66

Table 3. Nucleotide difference in *26S rRNA* sequences.

No.	Species	Nucleotide number*				
		22	86	89	94	204
1	*Kalanchoe* × *laetivirens*	G	A	G	G	T
2	*Kalanchoe daigremontiana*	A	.	.	.	.
3	*Dudleya viscida*	A	G	C	C	C
4	*Sedum nudum*	A	G	C	C	C
5	*Fendlera rupicola*	A	G	C	T	C
6	*Exbucklandia populnea*	A	G	C	C	C
7	*Mytilaria laosensis*	A	G	C	C	C
8	*Cornus officinalis*	A	G	C	T	C
9	*Disanthus cercidifolius*	A	G	C	C	C
10	*Cercidiphyllum japonicum*	A	G	C	T	C
11	*Phyteuma spicatum*	A	G	C	T	C

(*) Numbers referring to *Kalanchoe* × *laetivirens*.
(.) indicates that the nucleotide at a particular position is the same as the one of *Kalanchoe* × *laetivirens* sequence.
Bold nucleotides in the box are the critical nucleotides for the identification of *Kalanchoe* × *laetivirens.*

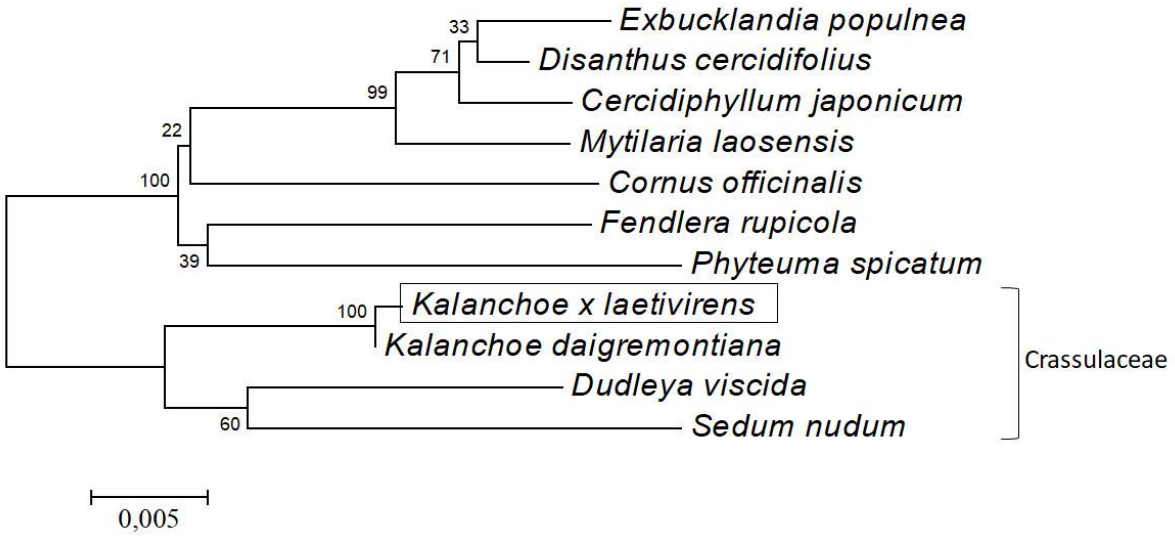

Figure 3. Dendrogram constructed on the basis of *26S rRNA* sequences by using the neighbor-joining method with 1000 bootstraps.

Kalanchoe × *laetivirens* and *K. daigremontiana* were the same, namely AGGT, whereas those in others were GC(C/T)C. The phylogenetic tree showed that *Kalanchoe* × *laetivirens* was closely related to *K. daigremontiana* with a bootstrap value of 100%. Both were in the Crassulaceae cluster together with *Dudleya viscida* and *Sedum nudum*. Other species belonging to different families were in a separate group (Figure 3).

DNA sequences analysis of *ITS*

The BLASTn analysis of *ITS* sequences showed that *Kalanchoe* × *laetivirens* had the highest similarity to *K. marineriana* with the identity value of 98.63%, query cover of 96%, and E-value of 0.0. The similarity level to *K. daigremontiana* was only 96.36% with a query cover value of 96% and E-value of 0.0 (Table 4). On the basis of the *ITS* sequence, three critical nucleotides could be applied to distinguish *Kalanchoe* × *laetivirens* from other studied accessions, namely, nucleotides 83 (in the ITS1 region), 264 (in the 5.8S rRNA region), and 644 (in the ITS2 region). At these positions, *Kalanchoe* × *laetivirens* had GTA. Meanwhile, in the other studied accessions, nucleotide 83 was deleted, and nucleotides 264 and 644 were AC (Table 5). The phylogenetic tree showed that *Kalanchoe* × *laetivirens* was in the same group as *K. daigremontiana* but was separated from three other *Kalanchoe* species (Figure 4).

Table 4. BLASTn alignment analysis of the *ITS* sequence of *Kalanchoe* × *laetivirens.*

No.	Species	Family	Query cover (%)	E-value	Identity (%)
1	*Kalanchoe marineriana*	Crassulaceae	96	0.0	98.63
2	*Kalanchoe rosei*	Crassulaceae	96	0.0	98.18
3	*Kalanchoe delagoensis*	Crassulaceae	96	0.0	97.88
4	*Kalanchoe fedtschenkoi*	Crassulaceae	96	0.0	97.12
5	*Kalanchoe beauverdii*	Crassulaceae	96	0.0	96.66
6	*Kalanchoe waldheimi*	Crassulaceae	96	0.0	96.66
7	*Kalanchoe daigremontiana*	Crassulaceae	96	0.0	96.36
8	*Kalanchoe bryophyllum*	Crassulaceae	96	0.0	95.76
9	*Kalanchoe gastonis-bonnieri*	Crassulaceae	96	0.0	95.94
10	*Kalanchoe rechingeri*	Crassulaceae	95	0.0	95.87

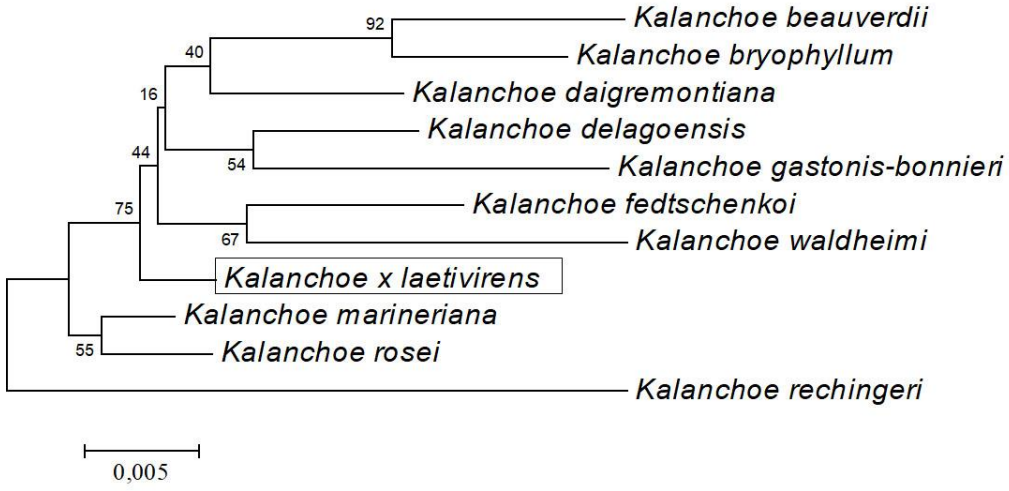

Figure 4. Dendrogram constructed on the basis of *ITS* sequences by using the neighbor-joining method with 1000 bootstraps.

Table 5. Nucleotide differences in *ITS* sequences.

No.	Species	Nucleotide number*		
		83	264	644
1	*Kalanchoe* × *laetivirens*	G	T	A
2	*Kalanchoe daigremontiana*	–	A	C
3	*Kalanchoe marineriana*	–	A	C
4	*Kalanchoe rosei*	–	A	C
5	*Kalanchoe delagoensis*	–	A	C
6	*Kalanchoe fedtschenkoi*	–	A	C
7	*Kalanchoe beauverdii*	–	A	C
8	*Kalanchoe waldheimi*	–	A	C
9	*Kalanchoe bryophyllum*	–	A	C
10	*Kalanchoe gastonis-bonnieri*	–	A	C
11	*Kalanchoe rechingeri*	–	A	C

(*) Numbers refer to *Kalanchoe* × *laetivirens*; (–) Deletion

Table 6. BLASTn alignment analysis of the *trnL-trnL-trnF IGS* sequence of *Kalanchoe* × *laetivirens.*

No.	Species	Family	Query cover (%)	E-value	Identity (%)
1	*Kalanchoe daigremontiana*	Crassulaceae	100	0.0	97.25
2	*Kalanchoe humilis*	Crassulaceae	92	0.0	97.05
3	*Kalanchoe gracilipes*	Crassulaceae	92	0.0	97.05
4	*Rhodiola rosea genotype Yakutia*	Crassulaceae	100	0.0	93.59
5	*Rhodiola rosea genotype Val de Nommon*	Crassulaceae	100	0.0	93.59
6	*Rhodiola rosea genotype Unteralp*	Crassulaceae	100	0.0	93.59
7	*Rhodiola rosea genotype Tonale*	Crassulaceae	100	0.0	93.59
8	*Rhodiola rosea genotype Piano dei Canali*	Crassulaceae	100	0.0	93.59
9	*Rhodiola rosea genotype Mattmark*	Crassulaceae	100	0.0	93.59
10	*Rhodiola rosea genotype Fedaia*	Crassulaceae	100	0.0	93.59

DNA sequence analysis of *trnL-trnL-trnF IGS*

The BLASTn analysis of the *trnL-trnL-trnF IGS* sequence showed that *Kalanchoe* × *laetivirens* had the highest similarity to *K. daigremontiana* with an identity value of 97.25%, query cover of 100%, and E-value of 0.0. The lowest similarity was found with the species of *Rhodiola* genus with an identity value of 93.59%, query cover value of 100%, and E-value of 0.0. All of the studied accessions belonged to the same family as *Kalanchoe* × *laetivirens*, namely Crassulaceae (Table 6). On the basis of the *trnL-trnF IGS* sequence, eight critical nucleotides distinguished *Kalanchoe* × *laetivirens* from other accessions: 508, 513, 523, 535, 539, 544, 545, and 558. At these positions, *Kalanchoe* × *laetivirens* had GGAAGTCA, whereas the other species had CATGAGTT. Nucleotides 508, 513, 523, and 535 were in the intron region of trnL (UAA), whereas the rest were in the IGS of trnL (UAA)3'exon-trnF (GAA). Five nucleotides in *Kalanchoe* × *laetivirens* were the same as those in *K. daigremontiana* but were different from those in the other studied accessions. Four of these nucleotides, i.e., 128, 242, 468, and 477, were in the intron region of trnL (UAA) and one of these nucleotides, i.e., 678, was in the intergenic spacer region of trnL (UAA) 3'exon-trnF (GAA). Furthermore, in the intron region of trnL (UAA), nine deletions were observed in *Kalanchoe* × *laetivirens* and *K. daigremontiana*, whereas other studied accessions had the same nucleotides, namely AAAAAGCG(A/C) (Table 7). The phylogenetic tree showed that *Kalanchoe*

× *laetivirens* formed a group with *K. daigremontiana* with a bootstrap value of 99%. Then, both clustered in the same group as fellow members of *Kalanchoe* and separated from the *Rhodiola* genus (Figure 5).

DNA sequences analysis of *ndhC-trnV IGS*

The BLASTn analysis of the *ndhC-trnV IGS* sequences showed that *Kalanchoe* × *laetivirens* had the highest similarity to *K. daigremontiana* with the identity value of 99.90%, query cover pf 100%, and E-value of 0.0. The lowest similarity index was found with *Sedum lineare* with an identity value of 87.25%, query cover of 100%, and E-value of 0.0. However, all the studied accessions belonged to the same family as *Kalanchoe* × *laetivirens*, namely Crassulaceae (Table 8). Based on the *ndhC-trnV IGS* sequences, one critical nucleotide, i.e., nucleotide 15 (located in

Table 7. Nucleotide differences in *trnL-trnL-trnF IGS* sequences.

No.	Species	Nucleotide number*																			
		1	1	1	1	1	1	1	1	1	2	4	4	5	5	5	5	5	5	5	6
		2	5	5	5	5	6	6	6	6	6	4	6	7	0	1	2	3	3	4	4	5	7
		8	6	7	8	9	0	1	2	3	4	2	8	7	8	3	3	5	9	4	5	8	8
1	*Kalanchoe × laetivirens*	T	–	–	–	–	–	–	–	–	–	A	T	G	G	G	A	A	G	T	C	A	T
2	*Kalanchoe daigremontiana*	.	–	–	–	–	–	–	–	–	–	.	.	.	C	A	T	G	A	G	T	T	.
3	*Kalanchoe humilis*	C	A	A	A	A	A	G	C	G	A	G	C	T	C	A	T	G	A	G	T	T	C
4	*Kalanchoe gracilipes*	C	A	A	A	A	A	G	C	G	A	G	C	T	C	A	T	G	A	G	T	T	C
5	*Rhodiola rosea genotype Yakutia*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
6	*Rhodiola rosea genotype Val de Nomnom*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
7	*Rhodiola rosea genotype Unteralp*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
8	*Rhodiola rosea genotype Tonale*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
9	*Rhodiola rosea genotype Piano dei Canali*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
10	*Rhodiola rosea genotype Mattmark*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
11	*Rhodiola rosea genotype Fedaia*	C	A	A	A	A	A	G	C	G	C	G	G	T	C	A	T	G	A	G	T	T	C
Region:		a													b							

(*) Vertical numbers show the nucleotide position referring to *Kalanchoe* × *laetivirens*.
(.) indicates that the nucleotide at a particular position is the same as the one of *Kalanchoe* × *laetivirens* sequence.
(−) Deletion.
Nucleotides in the box are the critical nucleotides for the identification of *Kalanchoe* × *laetivirens.*
(a) trnL(UAA) intron region.
(b) trnL(UAA)3'exon-trnF(GAA) intergenic spacer.

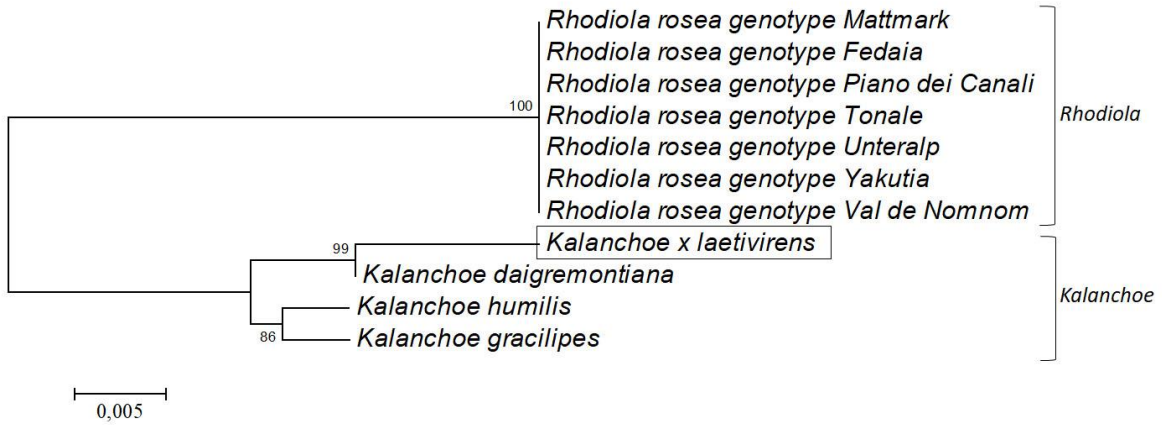

Figure 5. Dendrogram constructed on the basis of *trnL-trnL-trnF intergenic spacer* sequences by using the neighbor-joining method with 1000 bootstraps.

Table 8. BLASTn alignment analysis of the *ndhC-trnV IGS* sequence of *Kalanchoe* × *laetivirens.*

No.	Species	Family	Query cover (%)	E-value	Identity (%)
1	*Kalanchoe daigremontiana*	Crassulaceae	100	0.0	99.90
2	*Kalanchoe tomentosa*	Crassulaceae	100	0.0	96.66
3	*Phedimus kamtschaticus*	Crassulaceae	100	0.0	91.32
4	*Phedimus aizoon*	Crassulaceae	100	0.0	90.51
5	*Rosularia alpestris*	Crassulaceae	99	0.0	89.35
6	*Hylotelephium verticillatum*	Crassulaceae	100	0.0	88.60
7	*Hylotelephium ewersii*	Crassulaceae	100	0.0	88.73
8	*Umbilicus rupestris*	Crassulaceae	100	0.0	88.89
9	*Orostachys japonica*	Crassulaceae	100	0.0	87.54
10	*Sedum lineare*	Crassulaceae	100	0.0	87.25

Table 9. Nucleotide differences in *ndhC-trnV intergenic spacer* sequences.

No.	Species	Nucleotide number*														
		1	8	3	3	4	4	4	4	4	5	5	7	7	8	9
		5	1	2	4	7	7	7	8	8	2	2	1	5	0	0
				7	7	5	7	9	1	3	5	6	7	3	6	4
1	*Kalanchoe × laetivirens*	**T**	A	A	G	A	A	A	A	A	G	A	C	C	G	T
2	*Kalanchoe daigremontiana*	**C**	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	*Kalanchoe tomentosa*	**C**	G	T	T	T	T	T	T	T	T	G	A	A	T	C
4	*Phedimus kamtschaticus*	**C**	G	T	T	T	T	T	T	T	T	G	A	A	T	C
5	*Phedimus aizoon*	**C**	G	T	T	T	T	T	T	T	T	G	A	A	T	C
6	*Rosularia alpestris*	**C**	G	T	T	C	–	–	–	–	C	G	A	A	T	C
7	*Hylotelephium verticillatum*	**C**	G	C	T	C	T	T	T	–	T	G	A	A	T	C
8	*Hylotelephium ewersii*	**C**	G	C	T	C	T	T	T	–	T	G	A	A	T	C
9	*Umbilicus rupestris*	**C**	G	T	T	C	T	T	T	T	T	G	A	A	T	C
10	*Orostachys japonica*	**C**	G	C	T	C	T	T	T	–	T	G	A	A	T	C
11	*Sedum lineare*	**C**	G	T	T	C	G	G	G	T	T	G	A	A	T	C
Region:			a	b												

(*) Vertical numbers show the nucleotide position referring to *Kalanchoe* × *laetivirens*.
(.) indicates that the nucleotide at a particular position is the same as the one of *Kalanchoe* × *laetivirens* sequence.
(−) Deletion.
Bold nucleotides in the box are the critical nucleotides for the identification of *Kalanchoe* × *laetivirens.*
(a) *ndhC* gene.
(b) *ndhC-trnV intergenic spacer*.

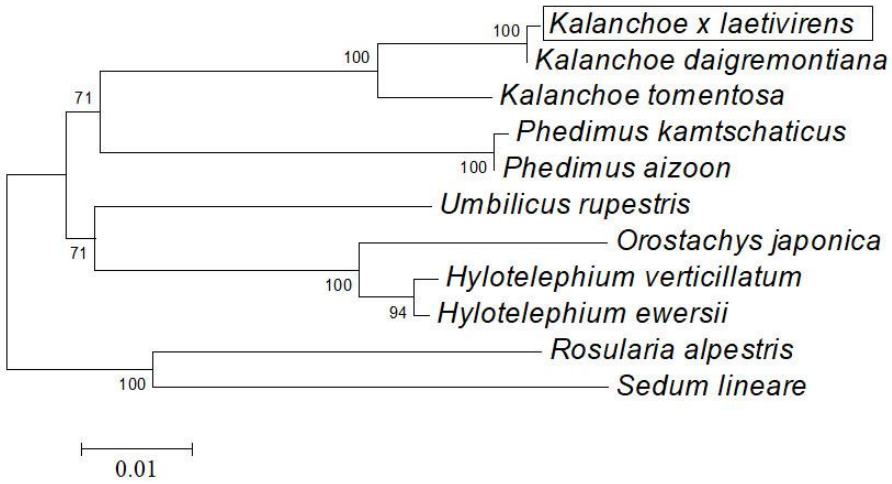

Figure 6. Dendrogram constructed on the basis of *ndhC-trnV intergenic spacer* sequences by using the neighbor-joining method with 1000 bootstraps.

the *ndhC* gene), could distinguish *Kalanchoe* × *laetivirens* from the other studied accessions. At that position, *Kalanchoe* × *laetivirens* had thymine, whereas other studied accessions had cytosine. Moreover, in the intergenic spacer region of ndhC-trnV, 14 nucleotides were similar between *Kalanchoe* × *laetivirens* and *K. daigremontiana* but were different from those in the other studied accessions (Table 9). The phylogenetic tree showed that *Kalanchoe* × *laetivirens* formed a group with *K. daigremontiana* with a bootstrap value of 100% and formed the same group as *K. tomentosa*. The *Kalanchoe* group was separated from other genus groups (Figure 6).

DNA sequence analysis of *rpl16 intron*

The BLASTn analysis of the *intron rpl16* sequences showed that *Kalanchoe* × *laetivirens* had the highest similarity with *K. daigremontiana* with the identity value of 97.80%, query cover of 100%, and E-value of 0.0. The lowest similarity was found in *Rhodiola dumulosa* with the identity value of 90.17%, query cover of 99%, and E-value of 0.0. All the studied accessions belonged to the same family as *Kalanchoe* × *laetivirens*, namely Crassulaceae (Table 10). The intron rpl16 sequences showed three critical nucleotides that could differentiate *Kalanchoe* × *laetivirens* from other studied accessions, i.e., 884, 912, and 923. At those positions, *Kalanchoe* × *laetivirens* had TTT, whereas the other accessions had CCC. Morever, at nucleotides 309–313 and 325, six deletions were observed in *Kalanchoe* × *laetivirens*, whereas TTTTAT was observed in the other studied accessions. Furthermore, 11 nucleotides in *Kalanchoe* × *laetivirens* were the same as those in *K. daigremontiana* but different from those in the other studied accessions. These nucleotides were 122, 144, 196, 249, 307, 566, 720, 740, 794, 834 and 873. From nucleotide 499 to nucleotide 506, eight positions were deleted, whereas TTCAAGAG were present in other studied accessions (Table 11). The phylogenetic tree showed that *Kalanchoe* × *laetivirens* formed a group with *K. daigremontiana* with a bootstrap value of 100% and grouped with *K. tomentosa*. This *Kalanchoe* group was separate from the *Rhodiola* and *Hylotelephium* groups (Figure 7).

Table 10. BLASTn alignment analysis of the *rpl16 intron* sequence of *Kalanchoe* × *laetivirens.*

No.	Species	Family	Query cover (%)	E-value	Identity (%)
1	*Kalanchoe daigremontiana*	Crassulaceae	100	0.0	97.80
2	*Kalanchoe tomentosa*	Crassulaceae	100	0.0	94.01
3	*Hylotelephium ewersii*	Crassulaceae	99	0.0	91.64
4	*Rhodiola humilis*	Crassulaceae	99	0.0	90.46
5	*Rhodiola sexifolia*	Crassulaceae	99	0.0	90.46
6	*Rhodiola prainii*	Crassulaceae	99	0.0	90.47
7	*Rhodiola rhodantha*	Crassulaceae	99	0.0	90.18
8	*Rhodiola ovatisepala*	Crassulaceae	99	0.0	90.45
9	*Rhodiola integrifolia*	Crassulaceae	99	0.0	90.26
10	*Rhodiola dumulosa*	Crassulaceae	99	0.0	90.17

Table 11. Nucleotide differences in *rpl16 intron* sequences.

No.	Nucleotide number*																										
	9	1 2	1 4	1 9	2 4	3 0	3 0	3 1	3 1	3 1	3 1	3 2	4 9	5 0	5 0	5 0	5 0	5 0	5 0	5 0	5 6	7 2	7 4	7 9	8 3	8 7	**8 8**	**9 1**	**9 2**
	0	2	4	6	9	7	9	0	1	2	3	5	9	0	1	2	3	4	5	6	6	0	0	4	4	3	**4**	**2**	**3**
1	–	C	A	C	T	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T	T	C	T	T	T	**T**	**T**	**T**
2	–	.	.	.	.	.	T	T	T	T	A	T	–	–	–	–	–	–	–	–	.	.	.	.	.	.	**C**	**C**	**C**
3	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	G	A	A	C	C	G	**C**	**C**	**C**
4	A	T	C	G	G	C	T	T	T	T	A	T	T	T	C	A	A	T	A	G	–	A	A	C	C	G	**C**	**C**	**C**
5	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**
6	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**
7	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**
8	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**
9	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**
10	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**
11	A	T	C	A	G	C	T	T	T	T	A	T	T	T	C	A	A	G	A	G	–	A	A	C	C	G	**C**	**C**	**C**

(1) *Kalanchoe* × *laetivirens*, (2) *K. daigremontiana*, (3) *K. tomentosa*, (4) *Hylotelephium ewersii*, (5) *Rhodiola humilis*, (6) *R. sexifolia*, (7) *R. prainii*, (8) *R. rhodantha*, (9) *R. ovatisepala*, (10) *R. integrifolia*, (11) *R. dumulosa*.
(*)Vertical numbers show nucleotide position referring to *Kalanchoe* × *laetivirens*.
(.) indicates that the nucleotide at a particular position is the same as the one of the *Kalanchoe* × *laetivirens* sequence.
(−) Deletion.
Bolded nucleotides in the box are the critical nucleotides for the identification of *Kalanchoe* × *laetivirens.*

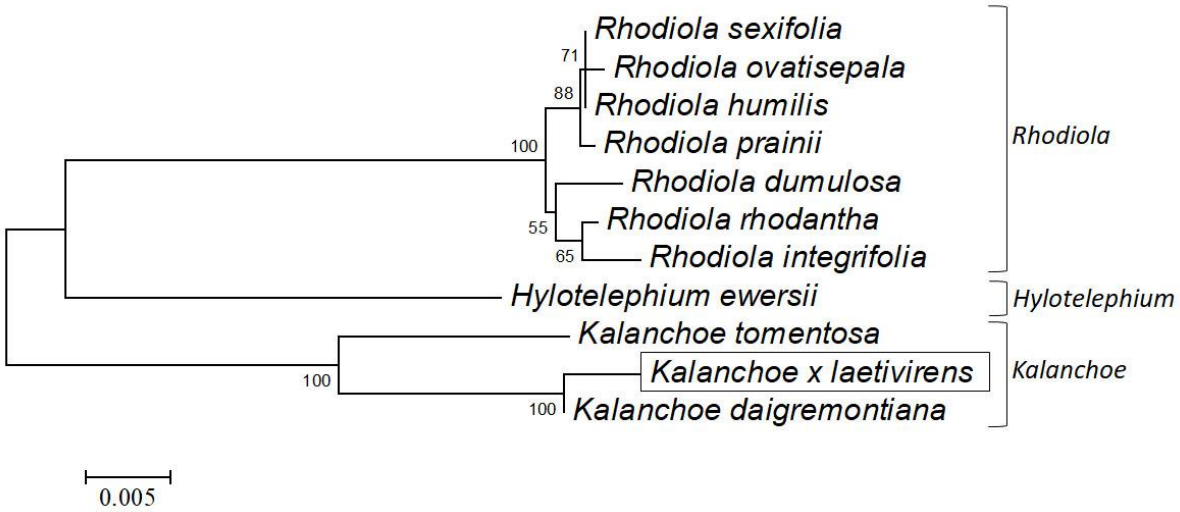

Figure 7. Dendrogram constructed on the basis of *rpl16 intron intergenic spacer* sequences by using the neighbor-joining method with 1000 bootstraps.

DISCUSSION

Cocor bebek is a member of genus *Kalanchoe*, which grows wild under various environmental conditions ranging from gardens to rocky soils. *Kalanchoe* can also be found in areas with tropical climates, such as Asia and Africa, and found abudantly in the southern African continent, especially Madagascar. Several well-known *Kalanchoe* species include *K. pinnata*, *K. blossfeldiana*, *K. delagoensis*, *K. daigremontiana*, and *K. mortagei* (Court, 2000). *K. daigremontiana* is one of the cultivated cocor bebek species and is often used as a female parent for cross hybridization with other species in this genus to produce a variety of hybrids. One

of the important hybrids that have been produced is *K. houghtonii* (a cross between *K. daigremontiana* and *K. delagoensis*) (Shaw, 2008).

Another species, namely *Kalanchoe* × *laetivirens*, was originally considered as a natural species. However, recent research has proven that *Kalanchoe* × *laetivirens* is a nothospecies, a hybrid between *K. daigremontiana* and *K. laxiflora* (Smith, 2020). Despite their different characteristics, *K.* × *laetivirens* is often mistaken for *K. daigremontiana*, which is one of its parents. The most basic difference between these species is that the leaves of *Kalanchoe* × *laetivirens* are completely green, whereas those of *K. daigremontiana* are green with purple bands on the lower surface (Shaw, 2008; Smith, 2020). Given that the said species is often misnamed, looking for a DNA barcode that can later be used for the molecular identification of this species is important. The DNA barcodes of each species are unique and unaffected by environmental conditions as long as they are available in a public database.

The alignment analysis of five DNA barcodes (Tables 2, 4, 6, 8, and 10) with BLASTn revealed no identity between *Kalanchoe* × *laetivirens* and *K. daigremontiana* that reached 100%. Therefore, those results indicated that they are different species.

No *K. laxiflora* DNA sequences were found in the GenBank database; however, *K. daigremontiana* DNA sequences were abundant in the database (updated 16 January, 2021). Therefore, in this study, the DNA sequences of *Kalanchoe* × *laetivirens* were compared only with those of one of its parents, namely, *K. daigremontiana*. High similarities between DNA sequences confirmed that *K. daigremontiana* is a parental genotype of *Kalanchoe* × *laetivirens*.

Sequence similarities between *Kalanchoe* × *laetivirens* and *K. daigremontiana* were found in four DNA barcodes, i.e., *26S rRNA* (four nucleotides), *trnL-trnF IGS* (five nucleotides), *ndhC-trnV IGS* (14 nucleotides), and *rpl16 intron* (11 nucleotides), as presented in Tables 3, 7, 9, and 11, respectively. A deletion was also found in the same position of nucleotides from both species. Meanwhile, the other examined accessions did not show any deletion. The nucleotide deletions observed in *Kalanchoe* × *laetivirens* and *K. daigremontiana* were found in *trnL-intron(UAA)* and *rpl16 intron* DNA sequences with nine (Table 7) and eight deletions (Table 11), respectively. The result of this study supported a previous finding showing that *Kalanchoe* × *laetivirens* is a nothospecies that is a derivative of *K. daigremontiana* (Smith, 2020). The offspring from cross planting between male and female parents contains a combination of both parent's genomes (Baek *et al*., 2018).

The five DNA sequences obtained in this study can be used as a DNA barcode for this species because critical nucleotides were produced in each sequence, i.e., 1, 3, 8, 1, and 3 critical nucleotides in the sequences of *26S rRNA* (Table 3), *ITS* (Table 5), *trnL-trnL-trnF IGS* (Table 7), *ndhC-trnV IGS* (Table 9), and *rpl16 intron* (Table 11), respectively. The most critical nucleotides were found in *trnL-trnF IGS*.

The critical nucleotides in *trnL-trnF IGS* were also found in small pandan (*Benstonea* sp.) from Riau with two critical nucleotides (Roslim, 2017) and durik-durik (*Syzygium* sp.) from Riau with as many as 53 critical nucleotides (Roslim, 2019). These results indicated that the *trnL-trnL-trnF IGS* sequence has considerable potential as a DNA barcode for many plant species considering that the spacer region has high mutation levels and high intraspecies variation. This region can be used to differentiate cryptic species with wide morphological variations (Shaw *et al*., 2007).

CONCLUSIONS

The five DNA barcodes, i.e., *26S rRNA*, *ITS*, *trnL-trnL-trnF IGS*, *ndhC-trnV IGS*, and *rpl16 intron*, obtained in this study

revealed high variations among the studied accessions due to the presence of substitution and deleted mutations. On the basis of nucleotide similarities in the five DNA barcodes, this study proved that *Kalanchoe* × *laetivirens* was a nothospecies with one of its parents being *K. daigremontiana*. The five DNA barcodes can be used as barcodes for the identification of *Kalanchoe* × *laetivirens* plants because they contain critical nucleotides.

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