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NEW DISTRIBUTION RECORDS OF *Rothmannia wittii* (RUBIACEAE) IN VIETNAM AND IDENTIFICATION OF DNA BARCODE SEQUENCE FOR *R. wittii*

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ABSTRACT

Using morphological comparison method, the authors identified that the specimens collected in Binh Chau-Phuoc Buu Nature Reserve is Rothmannia wittii. Information on the distribution of this species in Vietnam was also reported. Moreover, based on molecular biology *techniques, the matK and trnL-F regions of R. wittii were successfully amplified and sequenced. Also, the results showed that there were differences in matK and trnL-F sequences between R. wittii and R. daweishanensis that were similar in morphological characteristics.*

Keywords: *Rothmannia wittii, matK, trnL-F, DNA barcode.*

1. Introduction

Rothmannia Thunb. of the Rubiaceae is a genus of around 35 species occurring primarily in temperate regions of Asia, Africa, but with species also in the Asian tropics, especially in Southeast Asia (Bui & Nguyen, 2015). In Vietnam, the genus was known to include five species: *R. daweishanensis, R. eucodon, R kampuchiana, R. vietnamensis,* and *R. wittii* (Pham-hoang, 2000; Bui & Nguyen, 2015).

Rothmannia wittii (Craib) Bremek. was first described by Grab (1911), which the specimens were collected in Thailand. Newman et al. (2007) in the "A checklist of the Vascular Plant of Lao PDR" provided the information on the distribution of species in Laos. Recently, Bui & Nguyen (2015) recorded *R. wittii* for the flora of Vietnam, whose distribution was identified in Ninh Hoa district, Khanh Hoa province.

In 2018, the authors conducted some field trips to the Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc district, Ba Ria – Vung Tau province and encountered a flowering population of *Rothmannia* species. By comparison between its morphological attributes and those of reference species, it can be indicated that the *Rothmannia* species is *R. wittii.*

This study reported additional information on the distribution of this species in the southern region of Vietnam. The regions of *mat*K and *trn*L-F were previously used as

molecular markers for DNA barcoding of considerable number of species belonging to the family Rubiaceae and genus *Rothmannia* (Kainulainen & Bremer, 2014; Mouly et al., 2014). There is no information on DNA barcoding of *R. wittii*. In the present study, *mat*K and *trn*L-F were used for DNA barcoding and phylogenetic tree construction.

2. Materials and methods

2.1. Plant samples

Samples of *R. wittii* were collected from Binh Chau – Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc district, Ba Ria – Vung Tau province, location of about 10°21'01"N; 103°06'52"E, 33 m in elevation. Besides, *trn*L-F and *mat*K regions of some species of *Rothmannia* genus from GenBank were also used in phylogenetic analysis (Table 1).

Table 1. *Sequences from GenBank database used in this study*

Taxa	Accession number (*mat*K/*trn*L-F)	Taxa	Accession number (*mat*K/*trn*L-F)
R. venalis	KJ815792/ KJ815494	*R. urcelliformis*	KJ815791/ KJ815493
R. schoemannii	KJ815780/ KJ815483	*R. fischeri*	KJ815781/ KJ815484
R. merrillii	KJ815788/ KJ815490	*R. capensis*	KJ136897/ AM117384
R. daweishanensis	KJ815778/ KJ815481	*R. manganjae*	KJ815787/ KJ815489
R. macrosiphon	KJ815786/ KJ815488	*Alberta magna*	KJ136865/ FM207110
R. anna	KJ815777/ KJ815480		

2.2. Methods

2.2.1. Taxonomic method

Specimen was sampled and processed using conventional methods guided by the Royal Botanic Gardens, Kew (Bridson & Forman, 1999). Species identification was done using morphological vegetative and reproductive characteristics (Pham-hoang, 2000; Newman et al., 2007; Bui & Nguyen, 2015).

2.2.2. PCR amplification

Total genomic DNA was extracted from fresh leaf tissues using CTAB DNA extraction protocol (Huynh et al., 2018). The target *mat*K and *trn*L-F regions were amplified by polymerase chain reaction (PCR) using following barcoding primers (Table 2). The PCR reactions were performed in an Eppendorf Mastercycler Gradient using a volume of 25µl reaction mixture: 12.5µl Go-Taq green master mix (Promega, USA), 1.25µl of each forward and reverse primers (10 µM), 9.5µl nuclease-free water and 0.5µl DNA template (25µg/ml). PCR cycles consisted of an initial denaturation for 5 min at 95°C; 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55^{o}C) and extension (90 sec at 72°C); and a final extension at 72°C for 10 min. The PCR products were purified and sequencing by Nam Khoa Biotek Ltd. Company (Vietnam) using ABI 3130 XL Sequencer.

For multiple alignments, the ClustalW software (Thompson et al., 1994) was used to recognise the homology between sequences. Phylogenetic analysis was carried out with MrBayes (Ronquist & Huelsenbeck, 2003) using Bayesian methods with *Alberta magna* as the outgroup (Kainulainen & Bremer, 2014). Bootstrap values of 50% or higher were performed to obtain cluster supports. Besides, the Bioedit software was used to pairwise alignment using global alignment method between the DNA sequences of *R. wittii* and ***R.*** *daweishanensis*.

Table 2. *Primers used in the present study (Taberlet et al., 1991)*

Primers (*)/Region	Sequence (5'-3')
*trn*L-F (F)	CGAAATCGGTAGACGCTACG
*trn*L-F (R)	ATTTGAACTGGTGACACGAG
*mat*K (F)	ACCCAGTCCATCTGGAAATCTTGGTTC
*mat*K (R)	CGTACAGTACTTTTGTGTTTACGAG

(*) direction of primer F= forward, R= reverse

3. Results and discussion

3.1. Taxonomic treatment

Rothmannia wittii (Craib) Bremek. Bremek. 1957. Proc. Kon. Ned. Akad. Wetensch., C 60: 7; Puangsomlee. 2001. Nordic J. Bot. 21(2): 165-175; Bui & Nguyen. 2015. Jour. Bio. 37(4): 458-462. - *Randia wittii* Craib. 1911. Bull. Misc. Inform. Kew. 392. (Figure 1**)**

Tree 10–15m, glabrous throughout; gray-shaded; young twigs dichotomous. Leaf blade chartaceous, ovate, dark green above, pale green under side, midrib impressed adaxially and prominent abaxially, lateral veins diverging from the midrib and toward margin. Inflorescence uniflorous, protruding from a very reduced branch above a pair of leaves; pedicels 2–4 cm long, ca. 3 mm in diameter, green; Calyx lobes linear-oblong, 2–4 mm long, calyx tube cylindrical ca. 3 mm long; corolla tube glabrous, campanulate, white outside, purple inside, 3–5 cm long, ca. 3 cm wide; corolla lobes 5, glabrous, white outside, purple inside, 1.5 -2 cm long, ca. 1.5 cm wide. Fruit elongate to sub globose, 3–4 cm in diameter, dark green when young and black at maturity.

Studied specimens: H.T. Van 203, 8 August 2018, Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc District, Ba Ria-Vung Tau province, location of about 10°32'13"N; 107°26'55"E, 33 m in elevation.

Habitat: R. wittii grows on the low forest (about 30-40 meters in elevation), flowering from May to August and fruiting from July to December.

Distribution: R. wittii was formerly recorded in Thailand and Laos. In Vietnam, the species has been only recorded in Ninh Hoa district, Khanh Hoa province (Bui & Nguyen, 2015). This paper reported on *R. wittii* in Binh Chau – Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc district, Ba Ria – Vung Tau province.

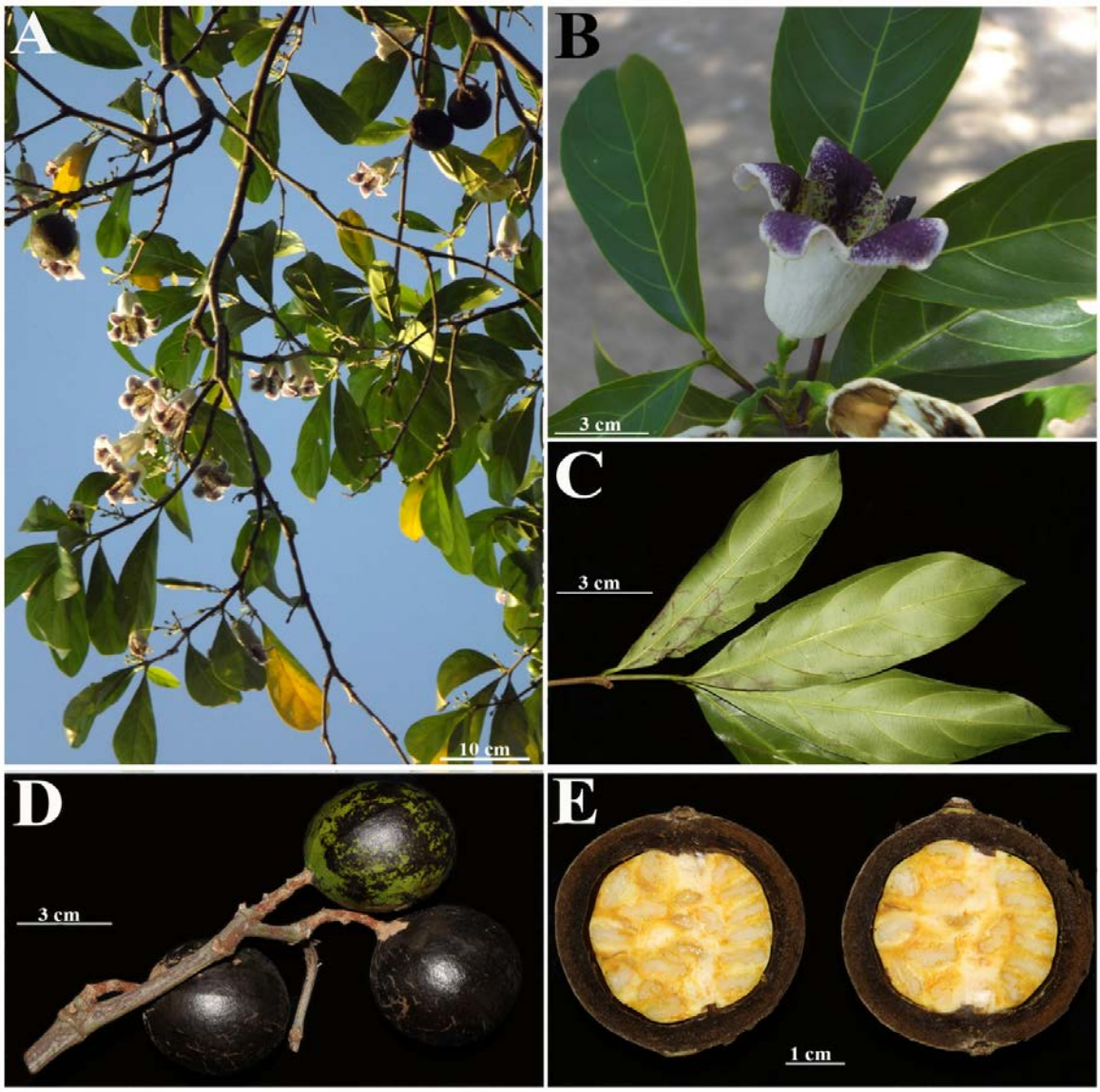

Figure 1. *Rothmannia wittii. A. Habitat, B. Flower and leaf blade (above side), C. Leaf blade (under side), D. Fruits, E. Longitudinal section of Fruit*

3.2. PCR amplification of trnL-F and matK

PCR products of *trnL-F* and *mat*K regions of the studied sample were clearly visualized on agarose gel in Figure 2 as sharp and big band with length about 900 bp and 800 bp, respectively. The results were consistent with expected sizes of PCR products amplified by primers by Taberlet et al. (1991).

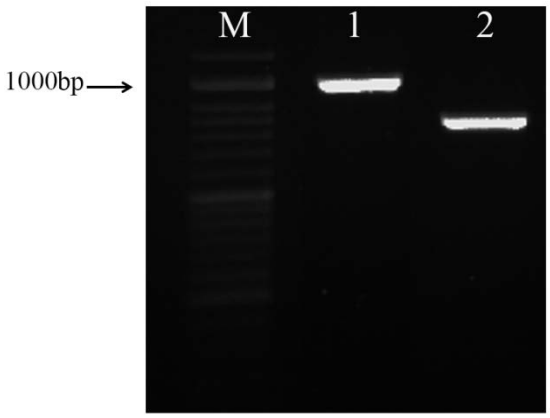

Figure 2. *PCR amplification result of trnL-F (1) and matK (2) regions of studied sample. M: ladder.*

3.3. Phylogenetic tree

The final lengths of the *trn*L-F and *mat*K sequences of studied sample were 804 and 760 bp, respectively. The average A+T content in the *trn*L-F and *mat*K regions were 65% and 66%, respectively.

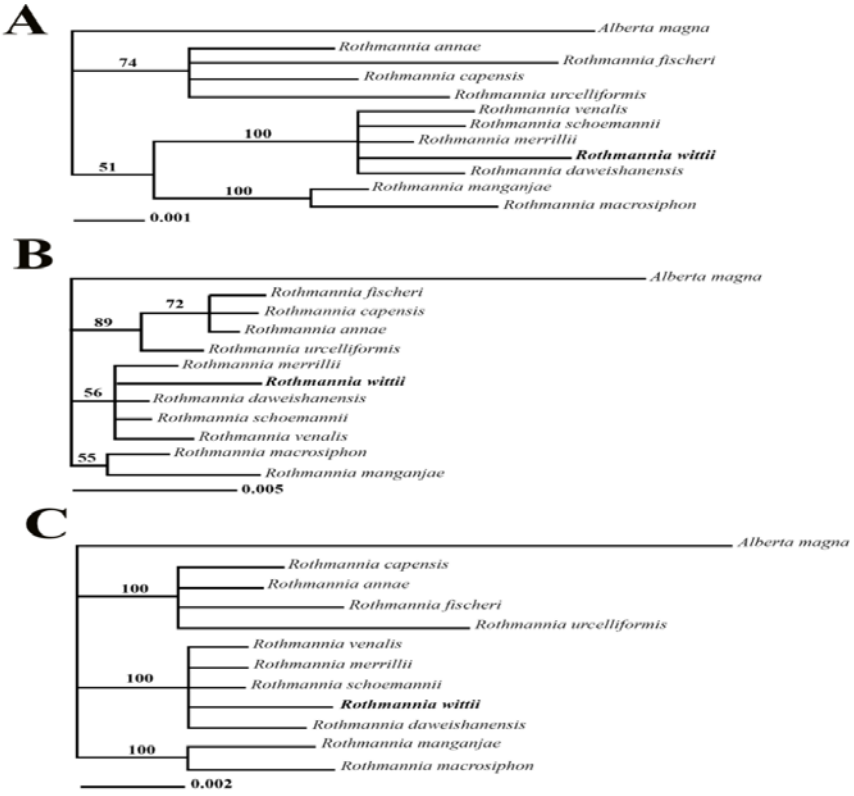

Figure 3. *Bayesian tree of 11 Rothmania species based on the trnL-F (A) region, matK region (B) and combination of trnL-F and matK regions (C). The bootstrap values of 50% or higher are shown above the nodes.*

By using *trn*L-F and *mat*K sequences in GenBank, the phylogenetic trees showed that relationship among *Rothmannia* species were established (Figure 3). Accordingly, the studied species (*R. wittii*) was group with *R. daweishanensis* with bootstrap value of 100%, 56% and 100% in *trn*L-F (Figure 3A), *mat*K (figure 3B) and combination of *trn*L-F and *mat*K regions (Figure 3C), respectively.

As mentioned above, *R. wittii* has not been classified via molecular markers yet. Moreover, there are only some studies which are used molecular markers to establish the phylogenic tree of Rubiaceae family whereas it is still lacking the study used molecular markers to establish the phylogenic tree of *Romania* genus. In previous study, Kainulainen and Bremer (2014) used some sequences of cpDNA (*mat*K, *rps*16, *rp*l32, *trn*T-F), rDNA (ETS), and nDNA (Xdh) to build the phylogenic tree of Rubiaceae family. Additionally, Mouly et al. (2014) also used molecular markers such as *trn*T-*trn*L, *trn*L-F, and *rps*16 to build the phylogenic tree of Rubiaceae family. The results showed that the distance between *R. wittii* and *R. daweishanensis* in the phylogenic trees (Figure 3A, B, C) are quite close. Note that, the arrangement of *R. wittii* and *R. daweishanensis* analyzed by molecular markers is similar that used by morphological analysis. Two species shared many of the same morphological characteristics: chartaceous, ovate leaf blade, uniflorous inflorescence, protruding from a very reduced branch above a pair of leaves, cylindrical calyx tube whereas the studied species was only distinguished from *R. daweishanensis* by 2-4 mm calys lobe (14-16 mm in *R. daweishanensis*). Therefore, the application of molecular markers is essential to distinguish these two species. In this paper, Bioedit software was used to compare sequence data of two molecular markers (*trn*L-*trn*F and *mat*K) of the studied species to those of closely related species in GenBank. These results were shown in Figures 4 and 5.

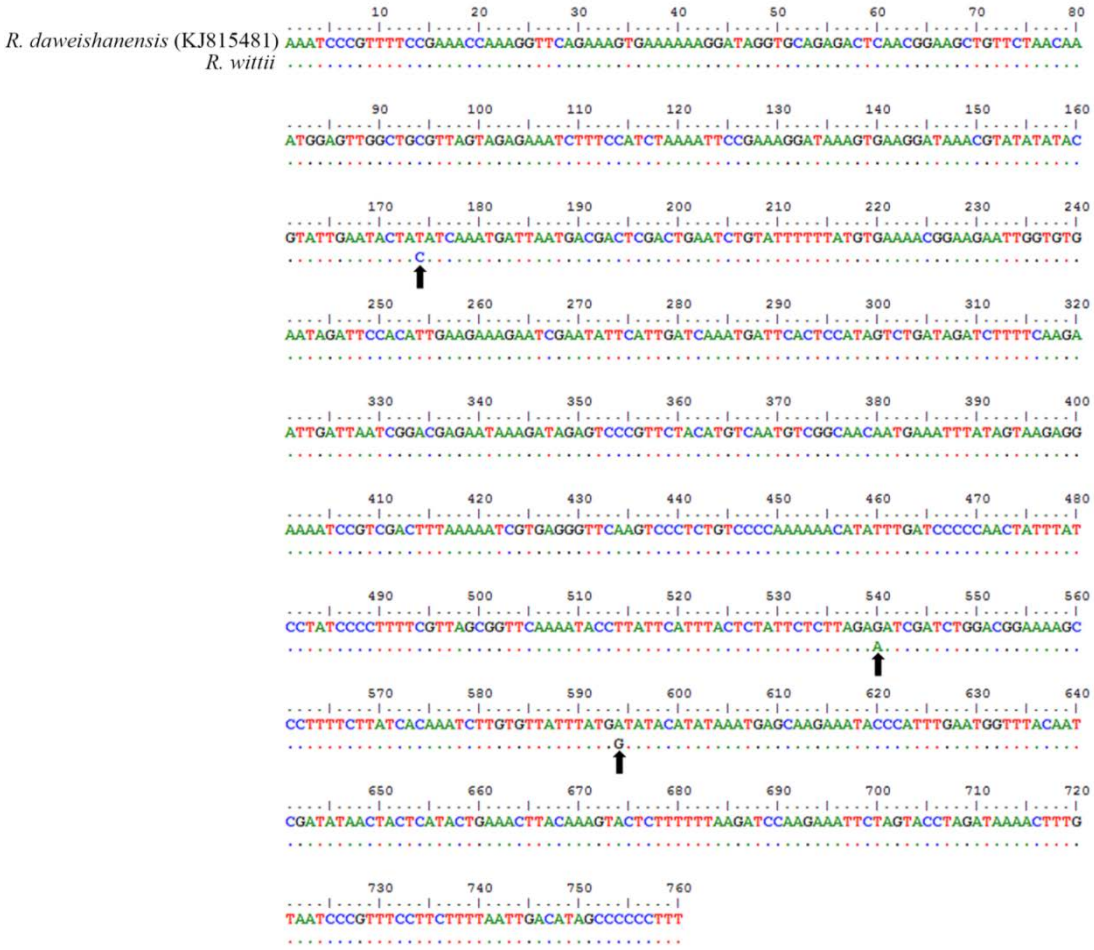

Figure 4. *The pairwise alignment of matK region between R. daweishanensis and R. wittii using Bioedit software. Note: the homologous positions (match) between 2 sequences are shown by the dots (.)*

The pairwise alignment of *mat*K region between *R. daweishanensis* and *R. wittii* (Figure 4) showed that the entire aligned length of *mat*K region of two specis is 760 bp. Two sequences have 757 homologous positions (match) and 3 non-homologous positions (mismatch) which located in positions 274, 540, 594, these mismatch positions were Cytosine, Adenine and Guanine in the sequence of *R. wittii* while Thymine, Guanine and Adenine were in the sequence of *R. daweishanensis*, respectively.

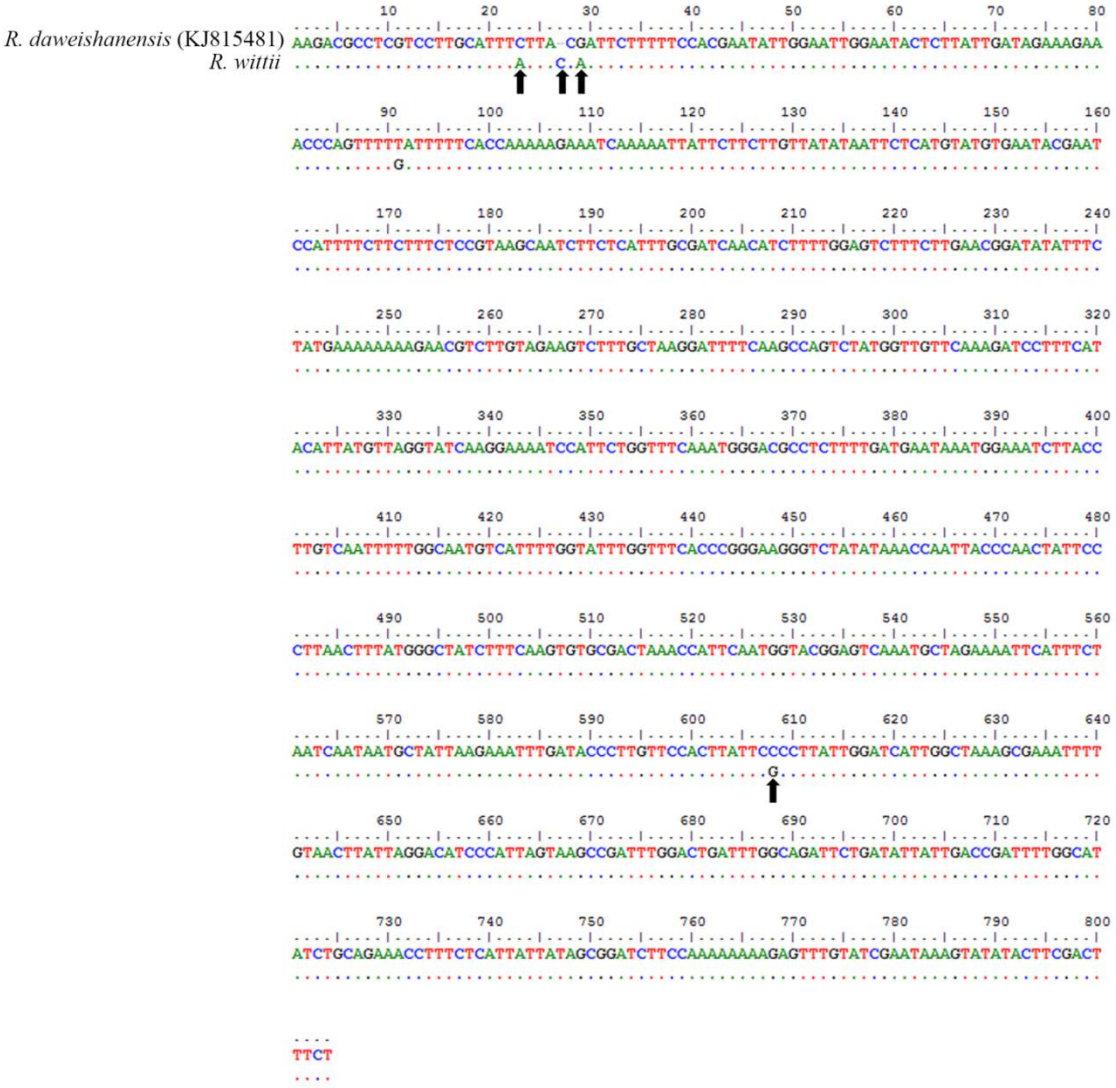

Figure 5. *The pairwise alignment of trnL-F region between R. daweishanensis and R. wittii using Bioedit software. Note: The homologous positions were presented as the dots (.), and the gap position was presented as the hyphen (-)*

The length of *trn*L-F region of *R. daweishanensis* was 803 bp. There was a gap at position 27 in the sequence of *R. daweishanensi pairwise aligned with the studied species R. wittii*. In addition, there were 3 mismatch positions (23, 29, and 608) between the sequences of *R. daweishanensis* and *R. witty* (Figure 5).

Recent reports have shown the importance of using the DNA barcodes to assist classification as well as establishment of the evolution of plant. In previous study, Ha & Nguyen (2015) used *mat*K region to DNA barcode of *Camellia tamdaoensis*, and the authors proved that there were differences in *mat*K region between *Camellia tamdaoensis* and *Camellia petelotii* whose morphological characteristics were identical. Moreover,

Nguyen et al. (2018) used ITS and *psb*A-*trn*H regions to DNA barcode of *Paris vietnamensis* in Vietnam, and the authors could distinguish this species from other species of *Paris* genus. In this study, based on comparison of *mat*K and *trn*L-F regions, the authors could identify the difference of genetic characteristics between *R. wittii and R. daweishanensis* which had similar morphological characteristics.

4. Conclusions

It was identified that the specimens of H.T. Van 203 collected from Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc District, Ba Ria-Vung Tau Province belonged to *R. wittii*. These results provided the additional information about its distribution in Vietnam. Moreover, the *mat*K and *trn*L-F regions of *R. wittii* were successfully amplified and sequenced. It was also proved that there were differences in *mat*K and *trn*L-F sequences between *R. wittii* and *R. daweishanensis* that had similar morphological characteristics.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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GHI NHẬN VÙNG PHÂN BỐ MỚI Ở VIỆT NAM VÀ XÁC ĐỊNH MÃ VẠCH DNA CHO LOÀI *Rothmannia wittii* (HỌ RUBIACEAE)

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TÓM TẮT

Bằng phương pháp hình thái so sánh, bài báo này đã xác định được mẫu nghiên cứu được thu tại Khu bảo tồn thiên nhiên Bình Châu – Phước Bửu là loài Rothmannia wittii, từ đó đã cung cấp thêm thông tin về vị trí phân bố mới cho loài này ở Việt Nam. Ngoài ra, bằng các phương nghiên cứu sinh học phân tử, nghiên cứu này cũng nhân bản và giải trình tự thành công vùng trình tự matK và trnL-F của loài R. wittii. Thông qua việc phân tích trình tự DNA, kết quả nghiên cứu đã cho thấy sự khác biệt trong trình tự vùng matK và trnL-F giữa 2 loài có đặc điểm hình thái tương tự là R. wittii và R. daweishanensis.

Từ khóa: *Rothmannia wittii, matK, trnL-F,* mã vạch *DNA barcode.*