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TAXONOMIC ASSESSMENT OF *CURCULIGO ORCHIOIDES* USING *mat*K AND *rbc*L DNA BARCODES

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SUMMARY

Curculigo orchioides is most commonly used as an imperative medicinal plant in Vietnam. The plant roots are mainly used to treat sexual dysfunction, back pain, arthritis, nephritis, jaundice, and infertility. Given the high market demand in the herbal market, *C. orchioides* traders commonly adulterate with other similar plants for illegitimate benefits. Maturase K (*mat*K) and ribulose 1,5-biphosphate carboxylase (*rbc*L) DNA barcode loci are presently used to identify the blends and counterfeit the medicinal herbs, as well as, identify the propagating conservation material. Nevertheless, the identification accuracy is highly dependent on NCBI Genbank or Barcode of Life Database (BOLD). In the recent study, the efficiency of DNA barcode loci, *mat*K and *rbc*L for the classification of *C. orchioides* populations, was investigated during 2020–2021. After examining 11 accessions of *C. orchioides* collected from different locations in Vietnam, the obtained results revealed that using NCBI database is more effective for classifying *C. orchioides*. In addition, the *mat*K locus also showed higher identification power than *rbc*L. The obtained findings could be helpful in the trading management, conservation, and development of *C. orchioides* in Vietnam.

Keywords: BOLD, classification, *Curculigo orchioides*, DNA barcodes, *mat*K, NCBI Genbank, *rbc*L

Key findings: The *mat*K and *rbc*L barcode loci were found as efficient tools for the classification of herbal plants. However, the accuracy depended on plant specificity and searched databases. Results revealed that the NCBI database was found to be better than the Barcode of Life database for classifying the *C. orchioides* populations. Furthermore, the discrimination power of *mat*K was found more superior than the *rbc*L locus.

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INTRODUCTION

Curculigo orchioides, commonly called golden eye-grass (also called 'sam cau' in Vietnam), is an endangered herbaceous flowering plant that belongs to the family Hypoxidaceae, and is mostly found in Southern China, Laos, Malaysia, Thailand, Philippines, and India. In Vietnam, this plant has been discovered in the mountainous provinces of Lai Chau, Tuyen Quang and Cao Bang up to the Central Highlands of Vietnam. It possesses high medicinal value and is habitually used to treat several diseases, such as, sexual dysfunction, back pain, arthritis, nephritis, jaundice, and infertility. Several remedial compounds from this plant have been reported, namely, phenolic glycosides, saponins and aliphatic

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compounds, cycloartan triterpenoid glycosides, and lycroine (Chauhan *et al*., 2010; Lamoral-Theys *et al*., 2010). These compounds exhibit strong medicinal properties, such as, anti-inflammatory potency, inhibit cetylcholinesterase, malaria resistance, cardiovascular protection (Lmoral-Theys *et al*., 2010), liver protection, anti-osteoporosis, antioxidant, and anti-cancer (Wang *et al*., 2017). Moreover, extracts from this plant have been shown effective in improving sexual behavior, i.e., penile erection, and mating performance in experimented rats (Chauhan *et al*., 2010). *C. orchioides* is also effective against cyclophosphamide-induced toxicity because of its antioxidant properties and regulation of inflammatory cytokine levels (Murali and Kuttan, 2015).

With the medical properties of *C. orchioides*, the demand for this medicinal herb is rapidly increasing. For this reason, there has been the phenomenon of mixing other plants with the same shape or color as *C. orchioides* to mislead the consumers. The traditional identification of this plant is based on the morphological characteristics of stems, leaves, and flowers. Some problematic issues encountered in applying this method, i.e., highly identical morphology features among plant species, variable polymorphisms between adult and juvenile stages, plant growth development phases, as well as, environmental factors, and all are leading to inaccurate classification. Furthermore, morphological identification cannot be performed properly if the specimen has been damaged or has been subjected to preliminary processing. The use of incorrect species with different pharmaceutical compounds would reduce the effectiveness of the medicine and could even harm the patient's health. The substitution of medical herbs could cause serious effects for the consumer, such as. allergies and reduced treatment effectiveness (Kumar *et al*., 2018).

Presently, different DNA based-methods have been developed to classify plant species. However, only Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) are mainly utilized for classifying the species in *Curculigo* genus (Babaei *et al*., 2012; Susindran and Ramesh, 2016; Hoang *et al*., 2018). Yet, these markers have some disadvantages, e.g., targeting random homologous genomic regions only or highly repetitive inter-simple sequence repeat for RAPD and ISSR, respectively (Mansour *et al*., 2010). DNA barcodes are popular in the identification of species in which standardized genomic regions are utilized to differentiate plant species. These DNA barcodes are used to serve the classification, biodiversity assessment, genetic resource conservation, and also to overcome the problem in morphology-based taxonomy. Different gene regions have been utilized as barcodes for plant classification, namely, ITS, *mat*K, *rbc*L, *atp*F-*atp*H, *psb*K-*psb*I, and *trn*H-*psb*A. However, *mat*K and *rbc*L are considered core barcode loci in plant classification.

Since the overexploitation and uncontrolled adulteration, *C. orchioides* was included in the Red Book of Vietnam and the Vietnam Red List of medicinal plants. Nevertheless, the studies to protect and develop this plant are scant, and most of the studies focus on *in vitro* propagation (Vo *et al*., 2011; Nguyen *et al*., 2018). Therefore, accurate classification of *C. orchioides* at the molecular level is essential for its further development and conservation. There have been no reported comprehensive studies using DNA barcodes to identify *C. orchioides*. Furthermore, the recognition accuracy of Genbank and BOLD databases, based on barcode sequences, varies depending on the plant species and DNA sequences availability in each database (Meiklejohn *et al*., 2019). Thus, the recent study compared the accuracy of NBCI Genbank and BOLD databases based on *mat*K and *rbc*L barcode loci for the genetic classification of *C. orchioides* and its related species collected in Vietnam. The obtained results will be useful for detecting adulteration, protection, genetic classification, and conservation of this important medicinal plant.

MATERIALS AND METHODS

Plant material

Leaves from a total of 11 plant accessions, including nine from species *C. orchioides* and two of their relatives, were collected from different locations in Vietnam (Figure 1), based on morphological description in the Vietnam Redbook (Nguyen *et al.,* 2007) and Gholave *et al.* (2021). The two relative accessions of *C. orchioides*, a so-called "sam cau do" in Vietnamese, were collected from Tay Ninh (TN) and Binh Dinh (BD) provinces and used as control plants. The control plant "sam cau do" accessions are morphologically similar to *C. orchioides*, but easy to grow and has lower medicinal value than *C. orchioides* and often an adulterant in *C. orchioides* trade (Nguyen *et al.,* 2020; Phan *et al.,* 2021). The samples of the accessions were kept in silica gel at room

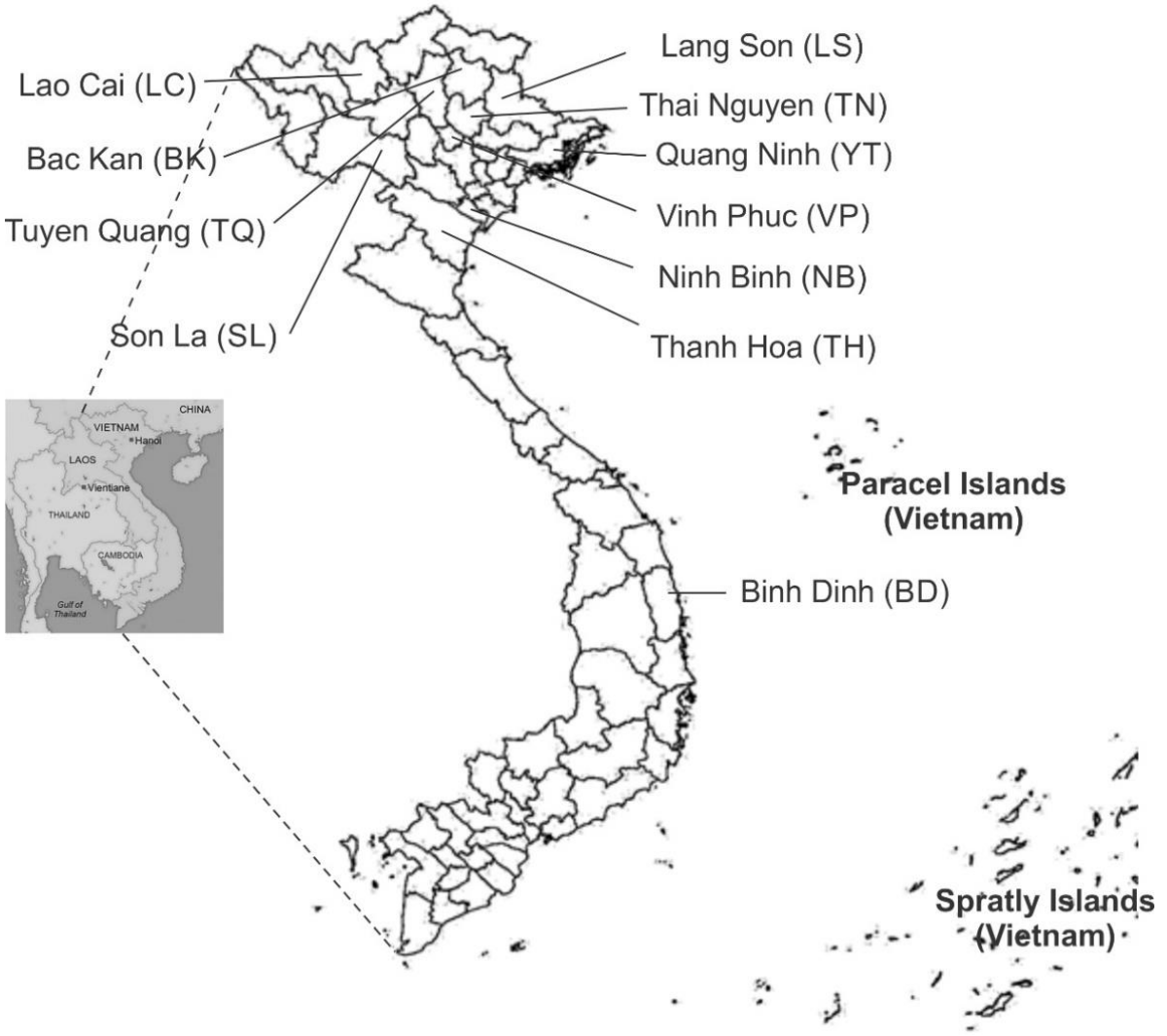

Figure 1. Targeted areas for collecting accessions in this study (Accession code collected in each location is indicated in parentheses).

temperature until dried during the years 2020–2021 at the Ho Chi Minh City University of Food Industry, Ho Chi Minh City, Vietnam. DNA from the dried leaves was extracted with the CTAB method as described by Doyle and Doyle (1987).

Methods

DNA regions of *mat*K and *rbc*L loci were amplified using polymerase chain reactions (PCR) with the reagent ingredients as follows: 7.5 µL 2X Mytaq Mix (Bioline, UK), 20 ng DNA, 0.2 µM primer (either *mat*K 390F: 5′-CGATCTATTCATTCAATATTTC-3′; 1326R: 5′-TCTAGCACACGAAAGTCGAAGT-3′ (13) or *rbcL*: cF: 5′- TGAAAACGTGAATTCCCAACCGTTT ATGCG-3′; and cR: 5′- GCAGCAGCTAGTT CCGGGCTCCA-3′ (Hasebe *et al*., 1994), where nuclease free water (Sigma-Aldrich, USA) was added to the final volume of 15 µL. The PCR cocktails were then amplified in a SureCycler 8800 Thermal Cycler (Agilent, USA) with thermal conditions as follows: initial denaturation at 95 °C in 2 min; then 35 cycles of 95 °C in 30 s, 55 °C in 30 s, and 72 °C in 1 min. This was followed by a 5 min extension step at 72 °C. The PCR products were then run on 1% agarose gel and using 1 kb DNA marker (Bioline, UK) to confirm the amplification length. The desirable PCR bands were excised, purified by ISOLATE II PCR and Gel Kit (Bioline, UK), sequenced using the BigDyeTM Terminator Cycle Sequencing Kit (Applied

Table 1. *C. orchioides* accessions collected for genetic characterization and corresponding accession numbers.

No.	Accession code	Collection site	Accession number for *mat*K gene	Accession number for *rbc*L gene
1	TN	Thai Nguyen	MW559808	MW559819
2	BK	Bac Kan	MW559809	MW559820
3	LC	Lao Cai	MW559810	MW559821
4	LS	Lang Son	MW559811	MW559822
5	SL	Son La	MW559812	MW559823
6	TQ	Tuyen Quang	MW559813	MW559824
7	TH	Thanh Hoa	MW559814	MW559825
8	NB	Ninh Binh	MW559815	MW559826
9	VP	Vinh Phuc	MW559816	MW559827
10	BD	Binh Dinh	MW559817	MW559828
11	YT	Quang Ninh	MW559818	MW559829

Biosystem, USA), and ran on ABI 3100 DNA analyzer (Applied Biosystem, USA). The obtained electropherograms were edited using FinchTV (Digital World Biology Products, USA) software. To increase sequence quality, noise signals were trimmed at both ends. Only sequences with PHRED scores >20 PHRED score were utilized for further analysis. Readings from both directions of each locus were combined by using merger online software from https://www.bioinformatics.nl/emboss-explorer/ to archive the final sequences. The obtained sequences were submitted to GenBank (NCBI, USA) and are publicly accessible under the accession numbers listed in Table 1.

The homology of *mat*K and *rbc*L sequences was concurrently checked with Basic Local Alignment Search Tools (BLAST) at http://blast.ncbi.nlm.gov using default parameters to identify taxonomy of accession at species level. The criteria for correct identification based on instructions from Nio *et al.* (2018) are the following: a sequence is considered correct if the highest identity percentage of searched sequences was derived from the expected species; otherwise, the identification was regarded as ambiguous when the highest identity percentage of searched sequences was not derived from the expected species, genus, and/or family. Simultaneously, the DNA sequences were identified by using the identification engine of BOLD system in the function of *rbc*L and *mat*K for plants with the link at https://www.boldsystems.org/index.php/IDS_OpenIdEngine. DNA sequences were then aligned with the ClustalW algorithm of the MEGA6 package (Tamura *et al*., 2013), using the default parameters. The alignment results were utilized to calculate the variable and parsimony informative sites among sequences. Evolutionary divergence for each data set and pattern of nucleotide substitution was performed by the same software. Evolutionary trees were constructed based on two methods consisting of Neighbor Joining (NJ) and Maximum Likelihood (ML) representing distance methods and discrete character methods, respectively (Kang *et al*., 2017). Kimura 2-parameter nucleotide substitution model was applied for both phylogenetic trees as this is one of the most widely used model for estimating genetic differences due to nucleotide substitution (Nishimaki and Sato, 2019). To estimate species resolution for a given barcode locus, we considered the species were resolved if conspecific individuals were grouped into one monophyletic branch in the phylogenetic tree with strong bootstrap support. Conversely, the species was considered as an identification failure if the same species individuals were separated in paraphyletic branches (Sikdar *et al*., 2018).

RESULTS AND DISCUSSION

Species identification

After both *mat*K and *rbc*L loci were successfully sequenced, the polymorphic informative sites and variable sites were computed for each locus. The aligned lengths were 704 bp and 671 pb for *mat*K and *rbc*L loci, respectively. Sequences of *mat*K locus show higher variable sites (7.8%) and parsimony informative sites (0.85) (Table 2). This finding is consistent with previous work as reported by Bhugwat *et al.* (2014), in which *mat*K region revealed higher variability than in *rbc*L in plants of the *Dalbergia* species.

Table 2. Summary statistics for potential barcode loci from 11 investigated accessions.

Locus	*mat*K	*rbc*L	*mat*K + *rbc*L
Number of sequences analyzed	11	11	11
Total number of sites	704	671	1375
Conserved sites	649 (92.2)	625 (93.1)	1274 (92.7)
Variable sites	55 (7.8)	46 (6.9)	101 (7.3)
Parsimony informative sites	6 (0.85)	3 (0.44)	101 (0.51)

Note: Values in parentheses are expressed in percentage.

Next, these sequences were targeted for homologous identification, the obtained sequences showing a minimum of 90 percent identity were considered. Using BLAST, both *mat*K and *rbc*L genes were showing identical results as described in Table 3. The classification results were consistent when using BLAST to search for the homology of both *mat*K and *rbc*L loci (Table 3). On the contrary, results from using BOLD were different. The returned identification from the BOLD database was not corresponding to those of BLAST.

Furthermore, the obtained results from BOLD were also not consistent between *mat*K and *rbc*L. The *mat*K recognition was more similar to those of BLAST with 10/11 accessions showing corresponding results. However, recognition results of BOLD on *mat*K locus were just able to identify up to the genus level. Moreover, the BD accession was incorrectly identified as *Molineria gracilis*. However, these results were completely different from the observations obtained from *C. glabrecens* of BLAST. Surprisingly, also using BOLD, none of the returned results from *rbc*L were similar to that of BLAST. Even more, all the returned identifications were *Hypoxis decumbens*, a distant species belonging to the genus *Hypoxis* in the family Hypoxidaceae.

Although a previous study highlighted the importance of the BOLD database in plant identification (Meiklejohn *et al*., 2019), the recent results showed the lower accuracy of BOLD in comparison with BLAST in classifying the *C. orchioides*. A likely explanation could be due to the small size and incompleteness of the BOLD database. Consequently, the missing species in the database cannot be identified and the method may assign the query sequence to an incorrect species (Parmentier *et al*., 2013). Incorrect species identification by BOLD was also reported by research on *Chenopodium murale* in Saudi Arabia (Bafeel *et al*., 2012).

Table 3. Search results of *mat*K and *rbc*L genes on the Genbank and BOLD databases.

No.	Accession code	Using BLAST with *mat*K sequences	Using BLAST with *rbc*L sequences	Using BOLD with *mat*K sequences	Using BOLD with *rbc*L sequences
1	TN	*Dracaena hokouensis*	*Dracaena hokouensis*	*Dracaena* sp.	*Ornithogalum bicornutum*
2	BK	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
3	LC	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
4	LS	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
5	SL	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
6	TQ	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
7	TH	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
8	NB	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*
9	VP	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo sp.*	*Hypoxis decumbens*
10	BD	*Curculigo glabrescens*	*Curculigo glabrescens*	*Molineria gracilis*	*Hypoxis decumbens*
11	YT	*Curculigo orchioides*	*Curculigo orchioides*	*Curculigo* sp.	*Hypoxis decumbens*

Estimation of sequence divergence

The divergence among the sequences was slightly variable (Table 4). The average pairwise distance of *mat*K was 0.034, almost two times higher than that of *rbc*L at 0.019. The divergence values of *mat*K and *rbc*L regions ranged from 0.0 to 0.174 and from 0.001 to 0.072, respectively. The TN accession collected from Tay Ninh province showed the highest difference at both *mat*K and *rbc*L barcodes. The substitution of different bases in analyzed regions was evaluated on entire codon positions (1^{st} + 2^{nd} + 3^{rd} nucleotide) and shown in Table 5.

In general, the transitional substitution was higher than the transversional substitution in both *mat*K and *rbc*L regions; whereas, the *rbc*L region exhibited a higher substitution rate than that of *mat*K. The recent findings were in agreement with the previous study presenting the higher variation of *mat*K region in comparison with *rbc*L in some plant species such as ferns (Li *et al*., 2011) and Rehmannia (Duan *et al*., 2019).

Table 4. Estimates of evolutionary divergence between DNA *mat*K and *rbc*L barcode sequences.

Accession code	TN	BK	LC	LS	SL	TQ	TH	NB	VP	BD	YT
TN	-	0.070	0.072	0.072	0.075	0.073	0.070	0.075	0.072	0.075	0.077
BK	0.168	-	0.001	0.001	0.004	0.007	0.000	0.008	0.001	0.011	0.006
LC	0.174	0.008	-	0.003	0.003	0.008	0.001	0.010	0.003	0.013	0.007
LS	0.168	0.000	0.008	-	0.006	0.007	0.001	0.008	0.003	0.011	0.006
SL	0.168	0.000	0.008	0.000	-	0.010	0.004	0.011	0.006	0.016	0.008
TQ	0.168	0.000	0.008	0.000	0.000	-	0.007	0.001	0.007	0.017	0.006
TH	0.168	0.000	0.008	0.000	0.000	0.000	-	0.008	0.001	0.011	0.006
NB	0.166	0.002	0.010	0.002	0.002	0.002	0.002	-	0.008	0.017	0.004
VP	0.166	0.002	0.010	0.002	0.002	0.002	0.002	0.000	-	0.013	0.006
BD	0.168	0.008	0.017	0.008	0.008	0.008	0.008	0.010	0.010	-	0.014
YT	0.168	0.000	0.008	0.000	0.000	0.000	0.000	0.002	0.002	0.008	-

The base substitutions per site from between sequences of *mat*K and *rbc*L regions is shown below and above the diagonal, respectively.

Table 5. The pattern of nucleotide substitution of *mat*K and *rbc*L regions (in percentage).

DNA Barcodes	*mat*K				*rbc*L			
Nitrogenous Bases	A	T	C	G	A	T	C	G
A	-	4.92	4.92	15.17	-	5.04	5.04	14.92
T	4.92	-	15.17	4.92	5.04	-	14.92	5.04
C	4.92	15.17	-	4.92	5. 04	14.92	-	5.04
G	15.17	4.92	4.92	-	14.92	5.04	5.04	-

Substitution patterns and rates were estimated under the Tamura-Nei model. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics.

Phylogenetic analyses

Based on phylogenetic analysis, 11 accessions were successfully classified into two main groups (Figure 2). Using either a single locus sequence or a combination of *mat*K + *rbc*L sequences reveals that TN accession (*Dracaena hokouensis*) was grouped in a separate branch, suggesting that these two DNA barcodes loci were highly promising to check the contamination of *Dracaena hokouensis* in herbal material. For identification of BD accession (*C. glabrescens*, a species in *Curculigo* genus), the classification was seemingly dependent on the method to develop the phylogenetic tree.

As presented in Figure 2, the NJ method is more applicable to separate this species from accessions belonging to *C. orchioides* species (Figure 2A, 2B, 2C) for both *mat*K and *rbc*L loci. However, if analysis with ML is applied, the adequate separation of BD accession was just found in *rbc*L (Figure 2E) and the combination of *mat*K and *rbc*L (Figure 2F). Thus, the single use of *mat*K locus by ML analysis should be avoided since BD was indistinguishable from several other accessions in *C. orchioides* species (Figure 2D).

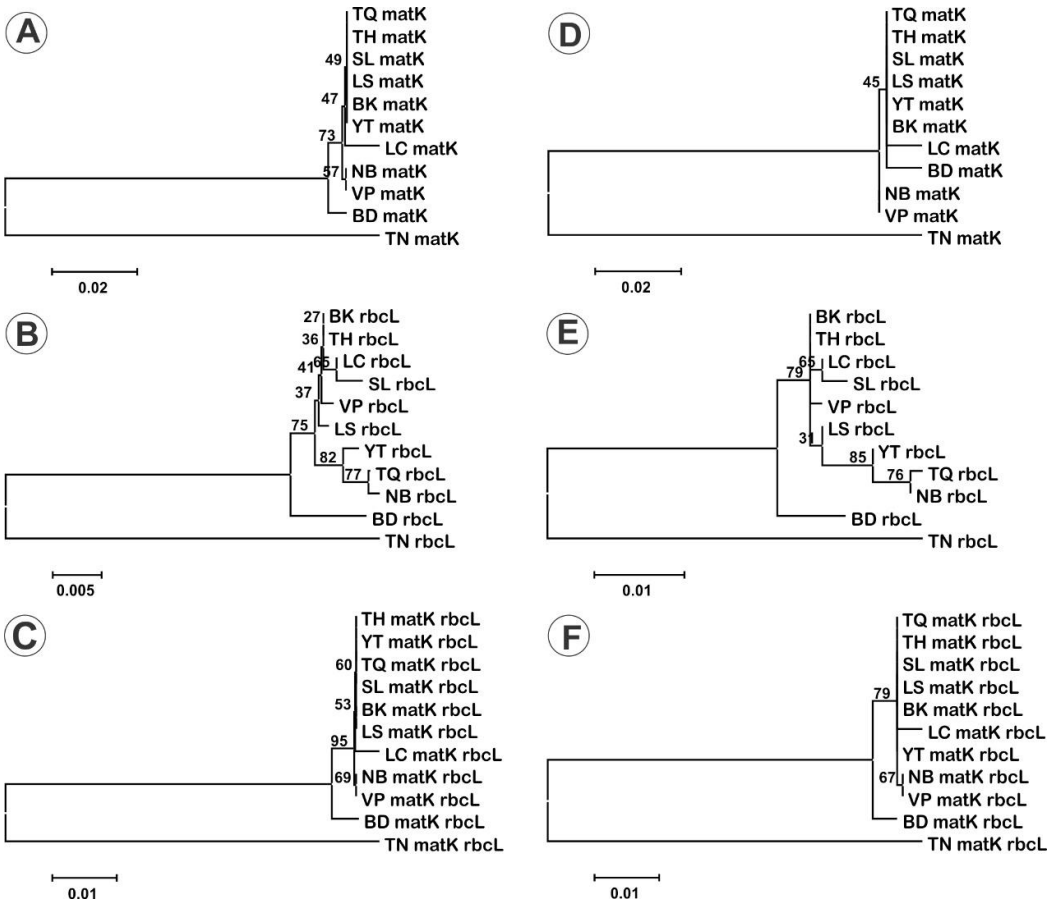

Figure 2. Phylogenetic tree based on *mat*K (A, D*), rbc*L (B, E), gene or the combination of *mat*K and *rbc*L (C, F) of 11 accessions by and Neighbour-Joining (A, B, C) and Maximum-Likelihood (D, E, F). (The value in the horizontal bar explains the length of the branch and represents the number of nucleotide substitutions).

The inconsistency in phylogenetic trees could be due to the differences in the algorithm of each method. The NJ is based on the distance method, whereas ML is based on the discrete character method. Past studies also reported the different phylogenetic results when the study was carried out in the family Dipterocarpaceae with data from *rbc*L and *mat*K genes (Harnelly *et al*., 2018). In this study, the combination of *mat*K and *rbc*L increased the grouping effectiveness of both analyses. These results were in line with the suggestion of the CBOL Plant Working Group, which proposed that the combination of multilocus barcodes could improve the classification of species (CBOL Plant Working Group, 2009). This combination was then continually supported by the following studies in different temperate flora (Burgess *et al*., 2011), *Hopea* species (Trang *et al*., 2015), and *Acacia* species.

CONCLUSIONS

The two most popular sequence databases consisting of NCBI Genbank and BOLD were used to distinguish the *C. orchioides* from its relative species based on nucleotide sequences of *mat*K and *rbc*L barcode loci. The obtained results proved the variable effectiveness of these two databases in identifying the species *C. orchioides*. However, the NCBI Genbank database was better than BOLD in terms of *C. orchioides* classification. Additionally, the discrimination of *rbc*L locus and the combination of *mat*K and *rbc*L were superior to *mat*K locus alone. Information obtained from this study could be useful in checking the adulteration of herbal medicines containing *C. orchioides* and applying them in the genetic conservation and development of this plant in Vietnam.

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