Identification of *Tinomiscium petiolare* from Vietnam using the DNA barcode

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Идентификация *Tinomiscium petiolare* из Вьетнама с помощью штрих-кода ДНК

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Background. *Tinomiscium petiolare* Hook.f. & Thomson is a medicinal species of the family Menispermaceae. This species is currently being intensively exploited for therapeutic purposes. Precise and rapid identification of *T. petiolare* is critical and essential for the classification, propagation, use and conservation of its genetic resources. In recent years, DNA barcoding has been known to be a fast and sensitive method for identifying species at any stage of development, using short DNA sequences. In this study we have performed the identification of *T. petiolare* specimens in Vietnam based on the sequence analysis of 4 DNA barcode loci: ITS, *matK*, *rbcL* and *rpoC*.

Materials and methods. Total DNA was extracted from leaf samples using DNeasy Plant Mini Kit. PCR amplification of the ITS, *matK*, *rbcL* and *rpoC* regions was carried out on the GeneAmp PCR System 9700 with specific primers. The purified PCR products were sequenced on the ABI 3500 Genetic Analyzer system, using BigDye®Terminator v3.1 Cycle Sequencing Kit. These genetic sequences were analyzed and compared, and a phylogenetic tree was constructed using BioEdit, BLAST, and MEGA 6 programs.

Results and conclusion. The success rate of amplification and sequencing was 100% for all 4 DNA barcode loci (ITS, *matK*, *rbcL* and *rpoC*) in the studied specimens. The produced sequence sizes of ITS, *matK*, *rbcL* and *rpoC* in the specimens were 574 bp, 810 bp, 527 bp and 488 bp, respectively. Further, we identified that all studied specimens were genetically related to each other and associated with the same species *T. petiolare*. Overall, the results of the study generated the most complete DNA barcode database of *T. petiolare* collected in Vietnam, contributing to the taxonomy and identification of this species.

Key words: Menispermaceae, sequence analysis, molecular identification, ITS, *matK*, *rbcL*, *rpoC*.

Актуальность. *Tinomiscium petiolare* Hook.f. & Thomson (тиномисциум черешковый) – лекарственный вид семейства Menispermaceae Juss. (Луносемянниковые). Этот вид в настоящее время активно используется в лечебных целях. Точная и быстрая идентификация *T. petiolare* имеет решающее значение для классификации, размножения, использования и сохранения его генетических ресурсов. В последние годы стало известно, что ДНК-штрихкодирование является быстрым и чувствительным методом идентификации видов на любой стадии развития с использованием коротких последовательностей ДНК. В этом исследовании мы провели идентификацию образцов *T. petiolare* во Вьетнаме на основе анализа последовательностей ДНК-штрихкодов 4 локусов: ITS, *matK*, *rbcL* и *rpoC*.

Материалы и методы. Тотальную ДНК экстрагировали из образцов листьев с помощью DNeasy Plant Mini Kit. ПЦР-амплификацию участков ITS, *matK*, *rbcL* и *rpoC* проводили в амплификаторе GeneAmp PCR System 9700 со специфическими праймерами. Очищенные продукты ПЦР секвенировали с помощью системы ABI 3500 Genetic Analyzer с использованием BigDye®Terminator v3.1 Cycle Sequencing Kit. Полученные последовательности были проанализированы, сравнены, и построено филогенетическое дерево с помощью программ BioEdit, BLAST и MEGA 6.

Результаты и выводы. Степень результативности амплификации и секвенирования составила 100% для ДНК-штрихкодов всех 4 локусов (ITS, *matK*, *rbcL* и *rpoC*) исследуемых образцов. Размеры последовательностей ITS, *matK*, *rbcL* и *rpoC* исследуемых образцов, которые мы получили, составляли 574 пн, 810 пн, 527 пн и 488 пн соответственно. Кроме того, мы определили, что все исследуемые образцы генетически связаны друг с другом и относятся к одному и тому же виду *T. petiolare*. В целом результаты исследования дали самую полную базу данных о ДНК-штрихкоде образцов *T. petiolare*, собранных во Вьетнаме, что способствовало идентификации и уточнению таксономии вида.

Ключевые слова: Menispermaceae, анализ последовательности, молекулярная идентификация, ITS, *matK*, *rbcL*, *rpoC*.

Introduction

Menispermaceae is a large family with about 68 genera and 440 species, including the genus *Tinomiscium* (Christenhusz, Byng, 2016). *Tinomiscium petiolare* is a medicinal species of the genus *Tinomiscium*. This species is found in China, India, Indonesia, Malaysia, Myanmar, Papua New Guinea, Philippines, Thailand, and Vietnam (Forman, 1988; Ho, 2000; Ghollasimood et al., 2012). The plants grow naturally and sparsely in mixed forests at elevations from 200 to 600 m. According to traditional medicine, *T. petiolare* has the effects on hemostasis, treatment of osteoarthritis pain, toothache, and cardiovascular disease (Van Valkenburg, Bunyapraphatsara, 2001; Chi, 2012).

Currently, *T. petiolare* is being intensively exploited for therapeutic purposes. Precise and rapid identification is critical for the classification, propagation, use and conservation of its genetic resources. Identification of this species is based on the morphological characteristics that have been studied (Ho, 2000; Chinh et al., 2015), but if the specimen is incomplete or crushed, it will be difficult to ensure high accuracy of morphological identification. Therefore, in order to overcome the disadvantages of morphology-based biological classification, the classification methods based on genetic materials have been studied and developed. In molecular classification techniques, the DNA barcode is a supporting tool for morphological classification (Hollingsworth et al., 2011). It is a modern technique that uses short DNA sequences to standardize differentiation between species (Kress, 2017). They have become a new tool to serve effectively in the inspection, classification, evaluation of genetic relationships, quality management, and origin of biological products (Mishra et al., 2015; Kress, 2017). In higher plants, some chloroplast genome regions (such as *matK*, *rbcL*, *psbA-trnH*, *atpF-atpH*, etc.) and nuclear genome regions (such as ITS-rDNA, 18S, etc.) are widely used in studying phylogenetic relationships, species taxonomy and identification (Kress et al., 2005; Mishra et al., 2015; Kress, 2017). However, for each different target group, the taxonomy of these genome regions was different (Kress et al., 2005; Tripathi et al., 2013).

Until now, the information about the DNA barcode of *T. petiolare* from Vietnam has not been studied. In this study, we carried out the identification of *T. petiolare* specimens on the basis of sequence analysis of 4 DNA barcode loci, namely ITS, *matK*, *rbcL* and *rpoC*. At the same time, this study also generated the database of DNA barcodes for *T. petiolare* plants collected in Vietnam for GenBank (NCBI..., 1988-2021).

Materials and methods

Plant materials

For this study, a total of 4 *Tinomiscium petiolare* samples were collected in Pu Luong Nature Reserve (PL1 and PL2) and Ben En National Park (BE1 and BE2) in Thanh Hoa province, Vietnam (Table 1). All these specimens were identified by Dr. Hoang Van Chinh (Hong Duc University) on the basis of their morphological characteristics. The specimens were deposited in the herbarium of Hong Duc University (Vietnam); 0.5 g of fresh leaves per plant sample were dried instantly in silica gel. All specimens were stored at –20°C until processed. Also, there were sequences downloaded from GenBank (Table 2).

DNA extraction, amplification and sequencing

Total DNA was extracted from leaf samples using DNeasy Plant Mini Kit (Qiagen, Germany). Amplification of the ITS, *matK*, *rbcL* and *rpoC* genes was carried out on the GeneAmp PCR System 9700 with the corresponding primers (Table 3). The PCR amplification reaction was performed in 25 µl reaction mixture consisting of 12.5 µl Master mix 2x (CWBIO, China), 0.75 µl each forward and reverse primer (10 pM/µl), 10 µl deionized water, and 1 µl of template DNA. The temperature cycle was as follows: one cycle of DNA denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with the last extension for 10 minutes at 72°C. The PCR product was tested on 0.8% agarose gel, then purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific Co., USA). The sequence of DNA fragments was determined on the ABI 3500 Genetic Analyzer system following Sanger's principle, with BigDye®Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., USA) by sequencing directly from PCR products.

Sequence alignment and analysis

DNA sequences were analyzed using BioEdit software (Hall et al., 2011). All positions that contain gaps and missing data were removed from the data set. The sequences of 4 specimens were compared with those published on GenBank (Table 2) using the BLAST tool (McGinnis, Madden, 2004). The sequences were registered on GenBank (see Table 1 for the accession numbers). Also, there were sequences downloaded from GenBank (Table 2). The genetic distance and phylogenetic tree were calculated and constructed using MEGA6 software (Tamura et al., 2013).

Table 1. Specimens for testing potential barcodes, and accession numbers in GenBank

Таблица 1. Образцы растений для исследования потенциальных штрихкодов и номера образцов нуклеотидных последовательностей в базе данных GenBank

Voucher number	Species name	Location	GenBank accession number			
			ITS	*matK*	*rbcL*	*rpoC*
PL1	*Tinomiscium petiolare*	Pu Luong, Thanh Hoa, Vietnam	MW147627	MW123076	MW123080	MW123084
PL2	*Tinomiscium petiolare*	Pu Luong, Thanh Hoa, Vietnam	MW147628	MW123077	MW123081	MW123085
BE1	*Tinomiscium petiolare*	Ben En, Thanh Hoa, Vietnam	MW147629	MW123078	MW123082	MW123086
BE2	*Tinomiscium petiolare*	Ben En, Thanh Hoa, Vietnam	MW147630	MW123079	MW123083	MW123087

Table 2. **Sequences from GenBank as extensions for species identification**

Таблица 2. **Последовательности из базы данных GenBank, использованные для видовой идентификации**

Species name	GenBank accession number			
	ITS	*matK*	*rbcL*	*rpoC*
Tinomiscium petiolare	HG004877, KY365658	HG005005, DQ478612	KF181577, EF173675	
Orthogynium sp.	KY365652			
Burasaia madagascariensis	KY365641			
Paratinospora sagittata	KY365666, KY365671, KY365672, KY365673			
Borismene japurensis		KC494024		
Burasaia apetala		KC494025		
Fibraurea tinctoria		KC494035	HQ260781, FJ026485	
Penianthus longifolius		KC494046	FJ026499	
Calycocarpum lyonii		KC494026		
Anamirta cocculus		KC494022	FJ626591, EU526983	
Sphenocentrum jollyanum			JN051687	
Tinospora sinensis				MN727386
Tinospora cordifolia				NC042153, MH577056
Sinomenium acutum				MN626719
Menispermum canadense				NC048451, MH298221
Menispermum dauricum				NC042371, MH298220

Table 3. **Primers used for amplification reactions in the study**

Таблица 3. **Праймеры, использованные для амплификации**

Locus	Primer name	Primer sequences (5′–3′)	Expected product length (bp)	Reference
ITS	ITS1F	TCCGTAGGTGAACCTGCGG	650	White et al., 1990
	ITS4R	TCCTCCGTCTATTGATATGC		
matK	*1RKIM*-f	ACCCAGTCCATCTGGAAATCTTGGTTC	900	Ki-Joong Kim, pers. comm.
	3FKIM-r	CGTACAGTACTTTTGTGTTTACGAG		
rbcL	*rbcLa*-f	ATGTCACCACAAACAGAGACTAAAGC	600	Levin et al., 2003
	rbcLa-r	GTAAAATCAAGTCCACCRCG		Kress, Erickson, 2007
rpoC	*rpoC*F	TGAGAAAACATAAGTAAACGGGC	570	Ford et al., 2009
	*rpoC*R	GTGGATACACTTCTTGATATTGG		

Results and discussion

Total DNA extraction and amplification of gene segments

Total DNA products after extraction and purification were examined by electrophoresis on 0.8% agarose gel. It can be seen that the obtained DNA samples were of good quality. The electrophoresis clearly exhibited only a DNA tape with high molecular weight, sharpness, and quality assurance for the next study steps.

Total DNA of the specimens, after its ultraviolet absorbance (OD) had been determined by a spectrophotometer, was diluted to the concentration required to template PCR with the specific primers (Table 3). The results of the electrophoresis of PCR products on 0.8% agarose gel showed that the primers ITS, *matK*, *rbcL* and *rpoC* were used successfully for amplifying desired gene segments from DNA of 4 specimens and subsequent fragment cloning. Specifically, PCR products in 4 sequence regions ITS, *matK*, *rbcL* and *rpoC* of 4 studied specimens had sizes of about 650 bp, 950 bp, 600 bp and 600 bp, respectively (Fig. 1). This result is consistent with the sequences of primers, sufficiently reliable as a basis for reading ITS, *matK*, *rbcL* and *rpoC* sequences in the specimens to serve for the next studies.

Sequence determination

After purification of PCR products, we performed ITS, *matK*, *rbcL* and *rpoC* gene sequencing in 4 studied specimens on the ABI 3500 Genetic Analyzer, using BigDye®Terminator v3.1 Cycle Sequencing Kit. The results showed that all four specimens were completely similar in all 4 sequences of ITS, *matK*, *rbcL* and *rpoC*. The consensus ITS, *matK*, *rbcL* and *rpoC* sequences for the 4 studied specimens were deduced as follows:

ITS

GTCGAATCGCAACCTTCTGGACGAGAGCCGGGCGGCCTCC
GCCTTCCCCGGTGCCTCGGCCGAAACAACAAACCCCGGCGCG
GCACGCGCCAAGGAAAACTCGAACGGAATTGGTGTGCCCGGAC
GATAGTGCATCGTCCCGGTGCCGCCGGTCTTCCGGGAAAATCTC
GAATGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGAT
GAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG
AATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCC
GAGGCCACCCGGCCGAGGGCACGTCTGCCTGGGCGTCACGC
GAACGCCGCTCCCGCCCCCTCGCAAGAGCGCGGACAGGAGC
GAACGTTGGCCCCCCGTGACCCGGCTCACGGTCGGCTTAAAAC
GGATCCCCCCTCGTTGCCCCGCGACGCGATCGGTGGTGGTTGAC
GGCAACCCTTACCCGCGATTGGACGACGCGACCGAGGGGCAC
GGGGGAAACGAACCCTTCGGAGAGAACTCCACGAGCGACCT
CAGGTCAGGCGGGGCCACCCGCTGAGTTTAA

matK

CTTTGCATTTATTGCGATTCTTTTTCTACGAGTATCATA
ATTGGAATAGGCTTATTACTCAAAAAAATAAATCCATTTCT
GTTTTTTTCAAAAAAAGAAAATCAAAGATTATTCTTT
GTTCCTATATAATTCTCATGTATATGAATGCGAATC
CATATTAGTTTTTTTCCGTAAACAATCTTTTTATTTACGATTAA
CATCTTCTAGAGCCTTTCTGGAGCGAACCCATTTCTATG
GAAAAATGGAACATCTTGTAGTAGTTTTTCAAAAC
GATTTTCAGTTTATCCTATGGTTGTTCAGGGAGCCTTTCATG
CATTATGTCAGATATCGAGGAAAATCCATTCTGGGTTCA
AAGGGGACCCTTCTTCTGATGAATAAATGGAACTATTACCTTG
TAAATTTCTGGCAATGTAATTTTGACTTGTGGTCTCAACTG
GATAGGATTTATATAACCCAATTAGCCAATCATTACTTC
GATTTTTTGGACTATCTTTCAAGTGTACGACTAAATACTTCGG
TAGTAAGGAGTCAAATGTTAGATAATTCATTTATTATGGATATT
GCGATTAAGAAGTTCGATAGTATAGTTCCAATTATTTCTTT
GATTGGATCATTGGCTAAAGCGAAATTTTGTAACGTAT
CAGGGCATCCCATTAGTAAGCCGGCTCGGGCCGATTCATCA
GATTCTGATATTATCGATAGATTTGGGCGAATATACAA
AAATCTTTCTCATTATTACAGCGGATCCTCAAAAAAAAA
TAGTTTGTATCGAATAAAGTATATACTTCGACTTTCCTGTGC
TAGAACTTTGG

rbcL

AAGATTACAAATTGACTTATTATACTCCTGACTATG
TACCCAAAGATACTGATACGCTAGCAGCATTCCGAGTAACTCCT
CAACCTGGAGTTCCGCCTGAAGAAGCGGGGGCTGCGGTAGCT
GCCGAATCTTCTACAGGTACATGGACAACTGTGTGGACCGATG
GACTTACCAGTCTTGATCGTTACAAAGGACGATGCTACGACATT
GAGCCCGTTGCTGGGGAAGAAAATCAATATATTTGTTATG
TAGCTTACCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTA
ATATGTTTACTTCCATTGTGGGTAATGTTTTTGGGTTCAAAGC
GCTACGCGCTCTACGTCTGGAGGATCTGCGAGTTCCTACTGCT
TATATTAAAACTTTCCAAGGCCCGCCTCATGGCATCCAAGTT
GAGAGAGATAAATTGAACAAGTATGGTCGTCCCCTATTGGGAT
GTACTATTAAACCAAAATTGGGATTATCCGCTAAGAACTACGG
TAGAGCAGTTTATGAATGTCTC

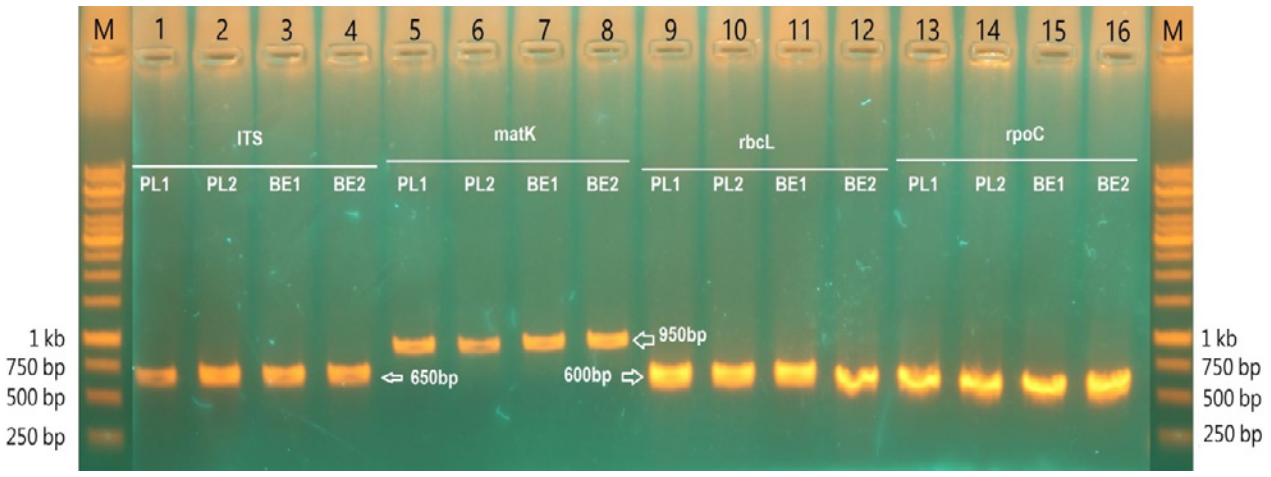

Fig. 1. DNA fragment amplification results for four studied specimens with primer pairs specific to the ITS, *matK*, *rbcL* and *rpoC* loci

Рис. 1. Результаты амплификации фрагментов ДНК четырех изученных образцов с парами праймеров, специфичных для локусов ITS, *matK*, *rbcL* и *rpoC*

rpoC

TTACAAGTCGTTTTCAGATGTAATTGAAGGCAAA
GAGGGAAGATTTCGCGAGACTCTGCTTGGCAAACGGGTC
GATTATTCGGGGCGTTCCGTCATTGTTGTGGGCCCTTC
GCTTTCATTAAATCGATGTGGATTGCCTCGCGAAATAG
CAATAGAGCTTTTCCAGACATTTGTCATTCGTGGTTTAAT
CAGACAACATATTGCTTCCAATATAGGGGTTGCTAAAAATA
AAATTCGGGAAAAAGAACCAATTGTGTGGGAAATAC
TTCAAGAAGTTATGCAGGGACATCCCGTATTGCTGAA
TAGAGCACCCACTCTGCATAGATTAGGCATACAGGCATTC
CAACCCATTTTAGTGGAAGGACGTGCTATTTGTTTACATC
CATTAGTTTGTAAGGGATTCAATGCAGACTTTGATGGGGAT
CAAATGGCTGTTCATGTACCTTTATCTTTGGAGGCT
CAAGCAGAGGCCCGTTTACTTATGTTTT

With 4 isolated sequences of ITS, *matK*, *rbcL* and *rpoC*, we identified 4 sequences of ITS, *matK*, *rbcL* and *rpoC* with the sizes 574 bp, 810 bp, 527 bp and 488 bp, respectively. Then, we analyzed and compared them with the ITS, *matK*, *rbcL* and *rpoC* sequences published in GenBank (NCBI http://www.ncbi.nlm.nih.gov/).

Sequence analysis

After obtaining the sequences of ITS, *matK*, *rbcL* and *rpoC*, we checked the similarity of the obtained sequences with those available in GenBank using the BLAST tool (McGinnis, Madden, 2004). In our study, the ITS, *matK* and *rbcL* sequences of the studied specimens showed a high similarity index corresponding to the ITS, *matK* and *rbcL* sequences of *T. petiolare* from GenBank. Specifically, the ITS sequence in the studied specimens showed a similarity index of 99.46–99.48% with the ITS sequence of *T. petiolare* from GenBank. The *matK* and *rbcL* sequences in the studied specimens showed a similarity index of 100% with the *matK* and *rbcL* sequences of *T. petiolare* in GenBank. Meanwhile, when compared with the available sequences from GenBank, the *rpoC* sequence of the studied specimens showed a similarity index of 98.16% with *T. sinensis*. This discrepancy might be due to the fact that the *rpoC* sequence data for *T. petiolare* is not currently available in `GenBank. The comparison with the GenBank database aimed to give a reference result with the group of species identical to the query nucleotide sequence. BLAST results could not lead to exact conclusions about species. In the cases of BLAST with high coverage and high homology (99%), it was not possible to revise the species name because BLAST results only showed the homogeneous nucleotide sequence that was available in GenBank. Since the results of BLAST showed inaccurate points, we conducted genetic distance determination and phylogenetic tree construction using MEGA6 software to determine the scientific names for the tested specimens (Tamura et al., 2013). The results are presented in Table 4 and Fig. 2.

The results in Table 4 demonstrate that the ITS, *matK*, *rbcL* and *rpoC* sequences of all the studied specimens collected in Ben En and Pu Luong (Thanh Hoa, Vietnam) had no differences. The ITS sequence of 4 studied specimens was not significantly different (0.13% and 0.4%) compared with the ITS sequence of *T. petiolare* published on GenBank with codes HG004877 and KY365658. Meanwhile, the difference between the ITS sequence that we obtained and the ITS sequences of some other species in the family Menispermaceae, published in GenBank with codes KY365652, KY365641, KY365673, KY365672, KY365671 and KY365666, was quite sizable. The *matK* and *rbcL* sequences produced from the studied specimens showed no difference when compared with *matK* and *rbcL* sequences of *T. petiolare* published

Table 4. Comparison of the studied gene sequences with the gene sequences in GenBank
(see Table 2 for the species name)

Таблица 4. Сравнительный анализ последовательностей изучаемых фрагментов и последовательностей, представленных в базе данных GenBank (см. видовые названия в таблице 2)

(a) ITS												
	BE1	**BE2**	**PL1**	**PL2**	**HG004877**	**KY365658**	**KY365652**	**KY365641**	**KY365673**	**KY365672**	**KY365671**	**KY365666**
BE1												
BE2	0.0000											
PL1	0.0000	0.0000										
PL2	0.0000	0.0000	0.0000									
HG004877	0.0013	0.0013	0.0013	0.0013								
KY365658	0.0040	0.0040	0.0040	0.0040	0.0013							
KY365652	0.1598	0.1598	0.1598	0.1598	0.1623	0.1715						
KY365641	0.1564	0.1564	0.1564	0.1564	0.1589	0.1679	0.0052					
KY365673	0.1645	0.1645	0.1645	0.1645	0.1671	0.1765	0.0200	0.0256				
KY365672	0.1645	0.1645	0.1645	0.1645	0.1671	0.1765	0.0200	0.0256	0.0000			
KY365671	0.1694	0.1694	0.1694	0.1694	0.1720	0.1818	0.0270	0.0327	0.0092	0.0092		
KY365666	0.1697	0.1697	0.1697	0.1697	0.1723	0.1820	0.0255	0.0298	0.0092	0.0092	0.0188	

Table 4. Continued
Таблица 4. Продолжение

(b) *matK*

	BE1	BE2	PL1	PL2	HG005005	DQ478612	KC494024	KC494035	KC494025	KC494046	KC494026	KC494022
BE1												
BE2	0.0000											
PL1	0.0000	0.0000										
PL2	0.0000	0.0000	0.0000									
HG005005	0.0000	0.0000	0.0000	0.0000								
DQ478612	0.0000	0.0000	0.0000	0.0000	0.0000							
KC494024	0.0150	0.0150	0.0150	0.0150	0.0150	0.0156						
KC494035	0.0188	0.0188	0.0188	0.0188	0.0188	0.0195	0.0137					
KC494025	0.0188	0.0188	0.0188	0.0188	0.0188	0.0182	0.0125	0.0163				
KC494046	0.0126	0.0126	0.0126	0.0126	0.0126	0.0131	0.0075	0.0075	0.0088			
KC494026	0.0241	0.0241	0.0241	0.0241	0.0242	0.0250	0.0267	0.0280	0.0293	0.0192		
KC494022	0.0277	0.0277	0.0277	0.0277	0.0277	0.0274	0.0355	0.0381	0.0342	0.0293	0.0280	

(c) *rbcL*

	BE1	BE2	PL1	PL2	KF181577	EF173675	FJ626591	JN051687	HQ260781	FJ026499	FJ026485	EU526983
BE1												
BE2	0.0000											
PL1	0.0000	0.0000										
PL2	0.0000	0.0000	0.0000									
KF181577	0.0000	0.0000	0.0000	0.0000								
EF173675	0.0000	0.0000	0.0000	0.0000	0.0000							
FJ626591	0.0057	0.0057	0.0057	0.0057	0.0057	0.0057						
JN051687	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0077					
HQ260781	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0076	0.0038				
FJ026499	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0077	0.0000	0.0038			
FJ026485	0.0019	0.0019	0.0019	0.0019	0.0019	0.0019	0.0076	0.0038	0.0000	0.0038		
EU526983	0.0057	0.0057	0.0057	0.0057	0.0057	0.0057	0.0000	0.0077	0.0076	0.0077	0.0076	

Table 4. The end
Таблица 4. Окончание

(d) *rpoC*												
	BE1	**BE2**	**PL1**	**PL2**	**MN727386**	**NC042153**	**MH577056**	**MN626719**	**NC048451**	**MH298221**	**NC042371**	**MH298220**
BE1												
BE2	0.0000											
PL1	0.0000	0.0000										
PL2	0.0000	0.0000	0.0000									
MN727386	3.9708	3.9708	3.9708	3.9708								
NC042153	3.9704	3.9704	3.9704	3.9704	0.0021							
MH577056	3.9704	3.9704	3.9704	3.9704	0.0021	0.0000						
MN626719	3.9660	3.9660	3.9660	3.9660	0.0237	0.0259	0.0259					
NC048451	3.9656	3.9656	3.9656	3.9656	0.0259	0.0281	0.0281	0.0021				
MH298221	3.9656	3.9656	3.9656	3.9656	0.0259	0.0281	0.0281	0.0021	0.0000			
NC042371	3.9656	3.9656	3.9656	3.9656	0.0259	0.0281	0.0281	0.0021	0.0000	0.0000		
MH298220	3.9656	3.9656	3.9656	3.9656	0.0259	0.0281	0.0281	0.0021	0.0000	0.0000	0.0000	

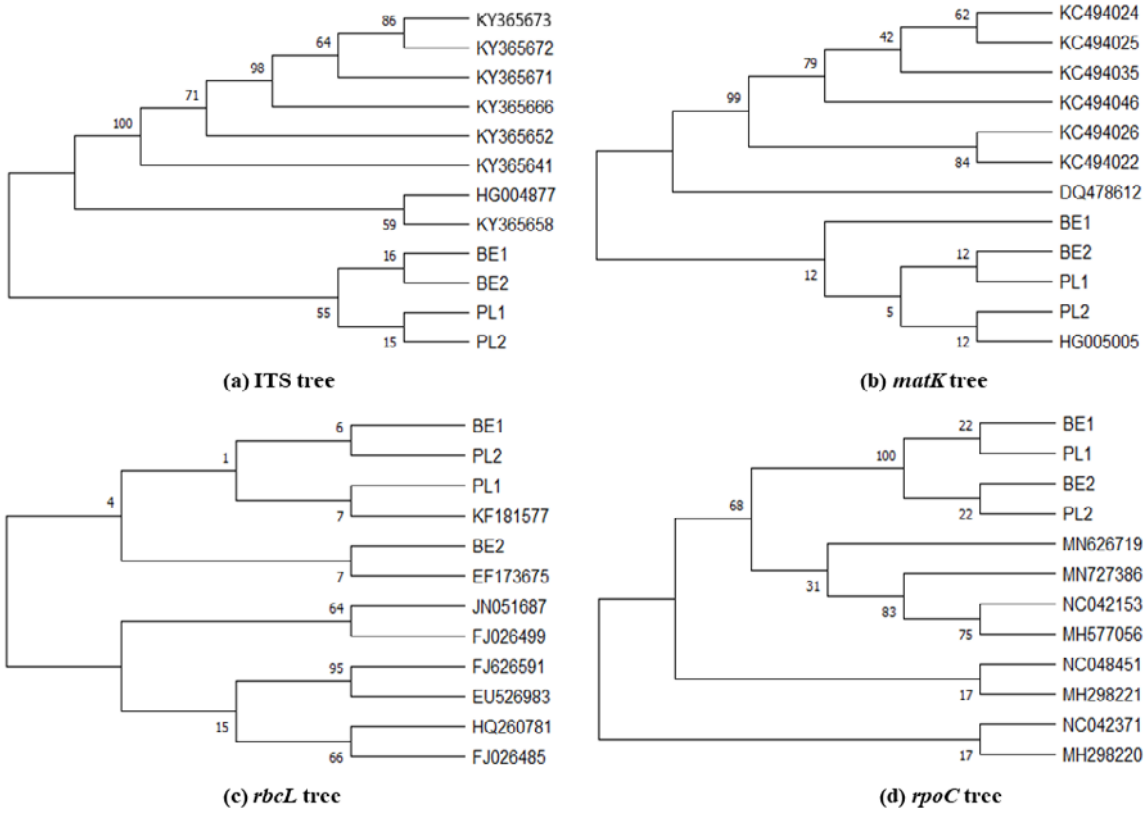

Fig. 2. **Tree diagram showing the genetic relationship between the studied gene sequences and the gene sequences published in GenBank** (see Table 2 for the species name)

Рис. 2. **Дендрограмма, показывающая родство последовательностей изучаемых фрагментов и последовательностей, представленных в базе данных GenBank** (см. видовые названия в таблице 2)

in GenBank with codes HG005005, DQ478612, KF181577 and EF173675. Meanwhile, the *matK* sequence that we obtained was different from the *matK* sequences of some other species in the same family Menispermaceae that were published in GenBank with codes KC494024, KC494035, KC494025, KC494046, KC494026 and KC494022. The difference rates were 1.50%, 1.88%, 1.88%, 1.26%, 2.41% and 2.77%, respectively. For the obtained *rbcL* sequence, there were differences with the *rbcL* sequences of some other species in the same family Menispermaceae published in GenBank with codes FJ626591, JN051687, HQ620781, FJ026499, FJ026485 and EU526983. The difference rates were 0.57%, 0.19%, 0.19%, 0.19%, 0.19%, and 0.57%, respectively. Notably, we did not find the *rpoC* sequence of *T. petiolare* on GenBank. So, the obtained *rpoC* sequence was significantly different from the *rpoC* sequences of other species in the same family Menispermaceae published in Genbank with codes MN727386, NC042153, MH577056, MN626719, NC048451, MH298221, NC042371 and MH298220. With these results, we confirmed that 4 studied specimens had genetic and species-specific relationships with *T. petiolare*.

Based on the identified sequences and homogenous ones from GenBank, we also built phylogenetic trees (Fig. 2). The results in Fig. 2 show that the ITS, *matK*, *rbcL* and *rpoC* sequences from the specimens that we collected and studied and the genetic sequence of *T. petiolare* were related to the same species and had a relatively high dissociation coefficient with other species. Therefore, the phylogenetic tree diagram demonstrates a diversity of branching, showing the genetic distance between the genetic sequence we obtained and the genetic sequences of some species that have been published in GenBank. The genetic sequences of the studied plant specimens collected at Pu Luong (PL1 and PL2) were not genetically different from those collected at Ben En (BE1 and BE2).

The results of the study have shown that the use of DNA barcodes is an effective method to quickly and accurately identify medicinal plant species (Chen et al., 2010; Mishra et al., 2016). Worldwide, there are also many studies that used DNA barcodes to identify species in the family Menispermaceae (Balasubramani, Venkatasubramanian, 2011; Yang et al., 2014; Osathanunkul et al., 2018; Wang et al., 2020). However, this study is the first to use a DNA barcode to authenticate *T. petiolare* from Vietnam. Of the four markers tested, ITS was arguably the most promising test region for species identification in this study. One advantage of the ITS region is that it can be amplified into two smaller segments (ITS1 and ITS2), therefore it is especially useful for degraded samples (Hillis, Dixon, 1991; Chen et al., 2010; Tripathi et al., 2013). The ITS region was also used to identify plant species with excellent results in previous studies (Balasubramani, Venkatasubramanian, 2011; Selvaraj et al., 2012; Thinh et al., 2020). At the same time, the results of this study also contribute to the DNA barcode database of *T. petiolare* in Vietnam.

Conclusions

We have succeeded in isolating and sequencing ITS, *matK*, *rbcL* and *rpoC* of the studied specimens collected at Pu Luong and Ben En (Thanh Hoa, Vietnam). The sequence sizes of ITS, *matK*, *rbcL* and *rpoC* that we obtained were 574 bp, 810 bp, 527 bp and 488 bp, respectively. The ITS, *matK*, *rbcL* and *rpoC* sequences of the studied specimens collected at Pu Luong and Ben En did not differ, showing that our specimens belonged to the same species group. The ITS, *matK*, *rbcL* and *rpoC* sequences of the studied specimens were identified as *T. petiolare*. For the first time, the *rpoC* sequence of *T. petiolare* was published in GenBank. The analysis also showed that there was still a lack of data on the genetic sequence of *T. petiolare* in other available databases than GenBank. Therefore, updating and supplementing molecular data about this species in order to have a sufficient basis for comparison and identification of plant samples is also a matter of concern and implementation.

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