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EVALUATION OF DNA BARCODES IN DISCRIMINATING *QUERCUS* SPECIES FROM LAM DONG, VIETNAM

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SUMMARY

DNA barcoding is a tool for species discrimination and identification which helps overcome the problem of identification that cannot be covered by morphological identification. *Quercus* is the second biggest genus of Fagaceae in Vietnam as well as Lam Dong province after *Lithocarpus*. However, the species discrimination study of the *Quercus* species in Vietnam and Lam Dong province has yet to be well uncovered due to ambiguous species boundaries and the lack of universal molecular markers. In this study, the DNA barcodes were tested to discriminate among the species of the *Quercus* genus in Lam Dong province. A total of sixteen and two samples of the *Quercus* and *Lithocarpus* genus (out-group) were tested using *mat*K, *rbc*L, and internal transcribed spacer (ITS). Of which, the new sequences in this study were sequenced from six species and one unknown species of *Quercus*, the rest was retrieved from GenBank. The BLAST, neighbor-joining, and Bayesian methods were employed to examine species discrimination success. The results showed that ITS was an efficient single-locus barcode for *Quercus* species by yielding the highest rate of universality as well as the best discriminatory and authentication power among the barcodes examined. In addition, the combination of ITS+*mat*K+*rbc*L achieved the highest species discrimination. Therefore, *mat*K and *rbc*L should not be used as DNA barcodes for the species identification of *Quercus*, whereas the combination of three genes that were proposed in this study is the most suitable DNA barcode for identifying *Quercus* species in Lam Dong province.

Keywords: DNA barcoding, ITS, Lam Dong province, *mat*K, *Quercus*, *rbc*L

INTRODUCTION

The *Quercus* genus with 531 species, belongs to the family Fagaceae (Hubert *et al*., 2014). Specimens of this genus are recognized by pendulous staminate inflorescences, carpellate flowers always solitary, capitate or dilated stigma and indehiscent cupules (Huang *et al*., 1999; Phengklai, 2008). In the genus, 250 species are known in the Americas, 125 species in Asia and 156 species in Europe, North Africa and Macaronesia (Govaerts, Flodin, 1998; Borazan, Babaç, 2003). Some species are often dominant in various forest types such as desert scrub forest, temperate deciduous forests and tropical montane forests (Nixon, 1993; Hubert *et al*., 2014; Valencia-A *et al*., 2016). *Quercus* species have been an importance role for economy, ecology and culture in many countries (Hubert *et al*., 2014). The taxonomic status of *Quercus* and its infrageneric classification have been widely discussed (Hickel, Camus, 1921; 1929; Camus 1935, 1935- 36; 1936; Govaerts, Flodin, 1998; Huang *et al*., 1999; Borazan,

Babaç, 2003; Ho, 2003; Ban, 2005; Newman *et al*., 2007; Phengklai, 2008). However, taxonomic treatments of *Quercus* species are still controversial because of restricted geographic distribution, treatment of species in regional floras, and morphological traits often exhibit broad ranges of intraspecific variation (Hubert *et al*., 2014). Using traditional taxonomy, the task of proper identification becomes arduous for the very closely related species. DNA barcode helps overcome the problem of identification that cannot be covered by morphological identification by using the genetic information. In the past few decades, many molecular markers have been developed to deal with the limitations of morphological and biochemical markers in *Quercus* species phylogenetics, such as chloroplast DNA (cpDNA: *rbc*L and *mat*K), and internal transcribed spacer (ITS) (Manos *et al.*, 1999; Manos *et al*., 2001; Piredda *et al.*, 2011).

In Vietnam, 51 *Quercus* species were reported, of which 43 species of *Quercus* were listed in "An illustrated flora of Vietnam" by Ho (2003) and "Vietnam plant checklist" by Ban (2005). Recently, the following eight species were reported: *Q. lineata* Blume (Li *et al.*, 2016); *Q. trungkhanhensis* Binh & Ngoc (Binh *et al.*, 2018a), *Q. bella* Chun & Tsiang. *Q. disciformis* Chun & Tsiang, *Q. xuanlienensis* Binh, Ngoc & Bon (Binh *et al.*, 2018b); *Q. baolamensis* Binh & Ngoc, *Q. bidoupensis* Binh & Ngoc, and *Q. honbaensis* Binh, Tagane & Yahara (Binh *et al*., 2018c). Lam Dong province is located in the Central highland of Vietnam where has long been known as one of the biodiversity hotspots in Vietnam and thirteen *Quercus* species were reported here (Ho, 2003; Binh *et al.*, 2018c). However, the species discrimination and phylogeny study of the *Quercus* genus in Vietnam and Lam Dong province have yet to be well uncovered due to ambiguous species boundaries and lack of universal molecular markers.

In this study, we selected three candidates of DNA barcodes (including *mat*K, *rbc*L, and ITS) for 10 species and one unknown species of *Quercus genus* distributed in Lam Dong Province. Our aims were (1) to examine the species discriminating ability of these three genes and using them as DNA barcodes for *Quercus* species in Lam Dong Province; (2) to evaluate the congruence of traditional taxonomic treatments for some closely related species based on morphological data and DNA barcoding results.

MATERIALS AND METHODS

Taxon sampling and outgroup selection

We studied 6 species and one unknown species of the genus *Quercus* collected from Lam Dong Province, Vietnam based on morphological differentiation (Table 1). Fresh mature leaves were collected from these *Quercus* species and immediately stored in silica gel in the field. The vouchers specimen with relevant information are listed in Table 1 and those specimens have been deposited in the herbarium of Dalat University (DLU). *Lithocarpus vuquangensis* and *Lithocarpus dahuoaiensis* were selected as outgroup for tree-based analysis (Ngoc *et al.*, 2016; Ngoc *et al.*, 2018). In addition, the sequences (including ITS, *mat*K, *rbc*L) of five *Quercus* species from Lam Dong Province which are available in GenBank of NCBI were also selected as barcode candidates in this study (Table 1).

Isolation of DNA, PCR amplification and sequencing

DNA extractions were performed by using the modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987), with minor modifications as in Toyama *et al*. (2016). Before the DNA extraction, we milled dry leaf material by QIAGEN TissueLyser to obtain fine powder. Then, the powder was washed up to five times by 1 mL buffer (0.1 M HEPES, pH 8.0, 2% mercaptoethanol, 1% PVP, 0.05 ascorbic acid). In this study, two regions of chloroplast genome (*mat*K and *rbc*L) and one nuclear ribosomal DNA (ITS) were amplified using the primer set as described in Table 2.

Table 1. List of vouchers specimens with GenBank accession number used in this study.

Taxon	Locality of voucher	Voucher No.	GenBank accession number		
			*rbc*L	*mat*K	ITS
Quercus baolamensis	Bao Lam District	V3191	LC318782*	LC318502*	MF770280*
Q. bidoupensis	Bidoup-Nui Ba National Park	V4328	LC318793*	LC318513*	MF770288*
Q. braianensis	Bidoup-Nui Ba National Park	QC171	MZ293773**	MZ293780**	MZ206335**
Q. braianensis	Bidoup-Nui Ba National Park	QC172	MZ293774**	MZ293781**	MZ206336**
Q. braianensis	Bidoup-Nui Ba National Park	V4445	LC318794*	LC318514*	MF770289*
Q. djiringensis	Di Linh District	V5537	LC318797*	LC318517*	MF770292*
Q. djiringensis	Di Linh District	V5538	LC318798*	LC318518*	MF770293*
Q. helferiana	Prenn Pass	QC 173	MZ293775**	MZ293782**	MZ206337**
Q. kerrii	Bidoup-Nui Ba National Park	QC174	MZ293776**	MZ293783**	MZ206338**
Q. lanata	Bidoup-Nui Ba National Park	QC175	-	MZ293784**	MZ206339**
Q. langbianensis	Langbian moutain	QC176	MZ293777**	MZ293785**	MZ206340**
Q. langbianensis	Bidoup-Nui Ba National Park	V3962	LC318790*	LC318510*	MF770285*
Q. langbianensis	Bidoup-Nui Ba National Park	V4165	LC318791*	LC318511*	MF770286*
Q. poilanei	Bidoup-Nui Ba National Park	V1895	LC318772*	LC318492*	MF770271*
Q. setulosa	Lang Hanh, Duc Trong District	QC177	MZ293778**	MZ293786**	MZ206341**
Q. sp	Bidoup-Nui Ba National Park	QC178	MZ293779**	MZ293787**	MZ206342**
L. dahuoaiensis	Da Huoai District, Lam Dong Province	V3194	LC318953*	LC318551*	KY436002*
L. vuquangensis	Vu Quang National Park, Ha Tinh Province	V5743	LC319671*	LC319670*	KY786083*

(*): From GenBank; (**): from this study; (-): Fail in sequencing.

Table 2. List of primers used in this study.

DNA region	Primer	Sequence (5' to 3')	Reference
*mat*K	*mat*K-XF	TAATTTACGATCAATTCATTC	Ford *et al*., 2009
	*mat*K-1326R	TCTAGCACACGAAAGTCGAAGT	Cuénoud *et al*., 2002
*rbc*L	*rbc*La-F	ATGTCACCACAAACAGAGACTAAAGC	Levin, 2003
	*rbc*L-724r	TCGCATGTACCTGCAGTAGC	Fay *et al*., 1997
ITS	ITS-18F	GTCCACTGAACCTTATCATTTAGAGG	Rohwer *et al*., 2009
	ITS-26R	GCCGTTACTAAGGGAATCCTTGTTAG	Rohwer *et al*., 2009

These three regions were amplified using universal primers of *mat*K, *rbc*L and ITS in 10 µL PCR reaction. The reaction components for effective PCR amplification are 1 µLof template DNA, 3.25 µL of Milli-Q water (Millipore), 0.5 µL 2xGflex PCR buffer (Mg2+, DNTP plus), 0.3 µLof each forward and reverse primer (10 pM) and 0.15 µLTks Gflex DNA polymerase (1.25 unit/µL). PCR amplification was conducted using these set of primers with the following program: for *mat*K template denaturation at 94°C for 5 min, followed by 38 cycles of denaturation at 94°C for 40 sec, annealing at 48°C for 40 sec., and extension at 72°C for 40 sec and a final extension of 7 min at 72°C; for *rbc*L and ITS template denaturation at 95°C for 4 min, followed by 29 (for *rbc*L) and 35 cycles (for ITS) of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min and a final extension of 10 min at 72°C. The amplification products were check by electrophoresis through 1.0% agarose gel, and then purified before DNA sequence analysis using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence editing and alignment

Based on the chromatogram's quality, the sequence editing process uses MEGA v7.0 Software (Kumar *et al.*, 2016). Sequence data must be free from noise and gaps to continue in the alignment process. The sequences were aligned using the Clustal W option in MEGA v7.0 Software (Kumar *et al.*, 2016). In this study, the new sequences together with thirty GenBank accessions were used to analyze. In addition, variable sites and parsimony information were also counted by using the MEGA v7.0.

Data analysis and Phylogeny inferences

Several methods have been used for the analysis of barcode data and species resolution. In which phylogenetic analysis, similarity approach as BLAST, and approaches based on the barcoding gap principle are the most commonly used for DNA barcode analysis (Tripathi *et al*., 2013). In this study, we employed similarity-based BLAST and tree-based methods to test the efficacy of barcode loci in the identification of *Quercus* species. Similarity-based BLAST is probably the most commonly used method practiced for classifying DNA sequences (van Velzen *et al*., 2012).

The rate of monophyletic groups in phylogenetic trees is often appropriate to evaluate the discriminatory power of a DNA barcode loci (Maia *et al*., 2012; Theodoridis *et al*., 2012; Zhang *et al*., 2012). Therefore, the phylogenetic trees were inferred based on the DNA data set of each gene (17 sequences of ITS, 17 sequences of *mat*K, and 16 sequences of *rbc*L) using Neighbor-joining (NJ) implemented in MEGA v7.0, the node support was assessed by a bootstrap test with 1000 pseudo-replicates of run with the K2P distance as a model of substitution. Two samples of *L. vuquangensis* and *L. dahuoaiensis* were used as an outgroup.

In addition, the combination of barcoding markers often performs better than single candidate DNA barcodes, and there are several combinations of markers were proposed in the past (Penisi 2007; Hollingsworth 2008). Hence, in this study, the three genes combined data set (19 DNA sequences of each gene, including outgroup) was used to infer the phylogeny tree by using the Bayesian method implemented in the program BEAST v1.10.4 (Drummond *et al.*, 2012) following the parameters set as described by Binh *et al.* (2018c).

RESULTS AND DISCUSSION

Rates of PCR amplification and sequence characteristics

In this study, a total of eight specimens representing 6 species and one unknown species of *Quercus* genus in Lam Dong province were obtained. All *Quercus* samples were successfully amplified and sequenced using universal primer pairs for three DNA barcoding regions (Table 2), except the *rbc*L region for one accession of *Q. lanata*. Three candidate barcodes (*mat*K, *rbc*L, ITS) were used to investigate the feasibility of DNA barcoding for species discriminating ability. The 23 new sequences (Table 1) obtained

from all markers, 24 sequences of *Quercus* genus and 6 sequences of outgroup (two species of *Lithocarpus* genus) from GenBank sequences to construct phylogenetic trees for *Quercus* species in Lam Dong province.

Among the species of *Quercus* in present study, the DNA sequences of *mat*K, *rbc*L, and ITS varied in length from 590 bp (*Q. lanata*) to 916 bp (*Q. braianensis*), 668 bp (*Q. braianensis*) to 727 bp (*Q. setulosa*), and 419 bp (*Q. langbianensis*) to 668 bp (*Q. sp*), respectively. The aligned data matrix of nuclear ITS sequences and cpDNA regions (*mat*K, *rbc*L) among 17 samples were shown some information comprising aligned length, variable sites, parsimony information and conserved (see Table 3 for details of the statistics).

Table 3. Statistics of datasets used for phylogenetic inference comprising *rbc*L, *mat*K and ITS sequences of eight samples of *Quercus* in this study and nine samples from GenBank.

Regions	*matK*	*rbcL*	ITS	Combined data
Percentage PCR success	100%	100%	100%	
Percentage sequencing success	100%	87.5%	100%	
Aligned length (bp)	859	671	488	2,018
Mean length (bp)	847.3	615.6	556.7	
Variable sites (bp)	13	4	64	81
Parsimony information (bp)	10	2	28	40

After the multiple sequence alignments, *mat*K produced the longest mean length of 847.3 base pairs (bp) followed by *rbc*L and ITS. Interestingly, ITS has the shortest mean length of 556.7 bp but possesses the highest number of parsimony informative characters with 28 from 64 variable sites (Table 3).

In the present study, three candidate loci (comprising *mat*K, *rbc*L and ITS) have 100% amplification success. However, while *mat*K and ITS have 100% sequencing success rates *rbc*L had the lowest sequencing success in these candidate loci with 87.5% (Table 3).

According to CBOL Plant Working Group (2009), an ideal DNA barcode is amenable to bidirectional sequencing with little requirement for manual editing of sequence traces and provide maximal discrimination among species. Thus, to measure the primers' universality, amplification and bidirectional sequencing success were assessed. Based on these criteria, both *mat*K and ITS performed well in terms of amplification and sequence quality, whereas *rbc*L showed good amplification, but had the lowest sequencing success in these trials. The single primer pairs for each of the three barcodes tested here performed without fail, as almost all samples of *Quercus* were successfully amplified and sequenced (Table 3). This indicated a very high universality for all the three DNA regions used in this study.

Phylogenetic relationship among *Quercus* species in Lam Dong Province

Three phylogenetic trees were reconstructed based on data set of *rbc*L, *mat*K, and ITS region (Fig. 1, 2, 3). Also, a Bayesian tree based on combined data set of three genes was also inferred (Fig. 4).

The Neighbor-joining tree of *rbc*L region (Fig. 1) was divided into two major clades (Clade I and Clade II). Clade I was supported by a low bootstrap value (58%) including fifteen samples representing 9 *Quercus* species except for V3191 (*Q. baolamensis*). In Clade II, *Q. baolamensis* is nested with two outgroup species (*L. dahuoaiensis* and *L. vuquangensis*) and supported by a weak bootstrap value (58%). In Clade I, there were eight species and one

unknown species. Here, interestingly, there four species which had more than one samples investigated in all including *Q. braianensis* (three samples), *Q. helferiana* (two samples), *Q. langbianensis* (three samples), and *Q. djiringensis* (two samples), while samples of *Q. langbianensis* were forming a monophyletic group, *Q. helferiana*, *Q. braianensis* and *Q. djiringensis* were not monophyletic. However, *Q. langbianensis* with individuals forming a monophyletic group in the trees with a bootstrap value under 50%. According to Zhang *et al.* (2012), only species with individuals forming a monophyletic group in the tree with a bootstrap value above 60% were accepted. On the other hand, *rbc*L trees cannot divide *Quercus* genus and *Lithocarcus* genus into different groups. Thus, species discriminating ability by *rbc*L region to identify *Quercus* species in Lam Dong Province is not good.

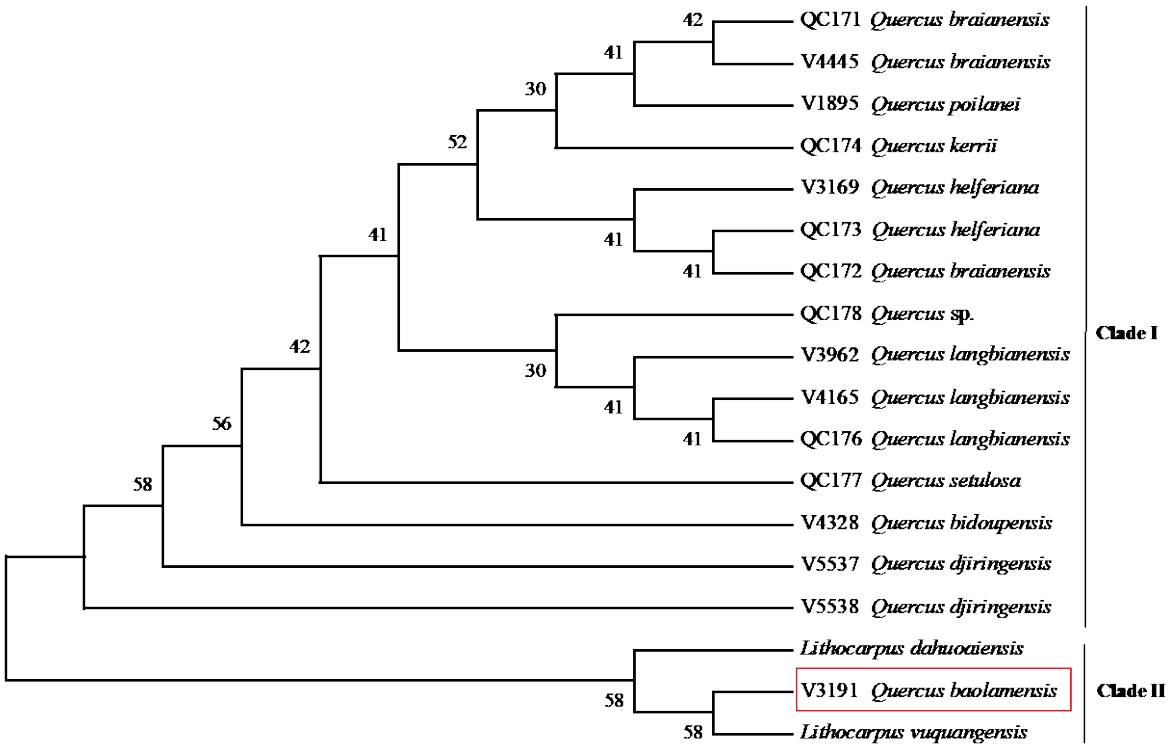

Figure 1. Neighbor-joining of *rbc*L sequences inferred using Kimura Two-parameter distances.

In the NJ trees of *mat*K and ITS region (Fig. 2, 3), both of them divided *Quercus* species into two major clades (Clade I and Clade II). In the *mat*K tree (Fig. 2), only the samples of *Q. langbianensis* formed a monophyletic group with a high bootstrap value 98%. In contrast, the samples of *Q. braianensis*, *Q. djiringensis*, *Q. helferiana* did not fall in monophyletic clades.

In the ITS tree (Fig. 3), the samples of *Q. braianensis*, *Q. helferiana*, *Q. langbianensis* and *Q. djiringensis* formed monophyletic groups with a bootstrap value of 65%, 34%, 53%, and 92%, respectively. In general, in the tree of NJ method, a species was considered to be resolved if the accessions under the species form a monophyletic group. In this study, the *Quercus* species discrimination was the highest for ITS, while *mat*K provided the second-highest species and followed by *rbc*L. This can be explained based on previous reports about the nuclear ITS which is regarded as one of the most appropriate DNA barcode regions because of its higher variability, which can distinguish even closely related species. (Chen *et al.*, 2010; Hollingsworth 2011;

Piredda *et al*., 2011). In contrast, plastid barcodes such as *mat*K, *rbc*L are usually more efficient when applied to a sampling of distantly related species (Alves *et al*., 2014).

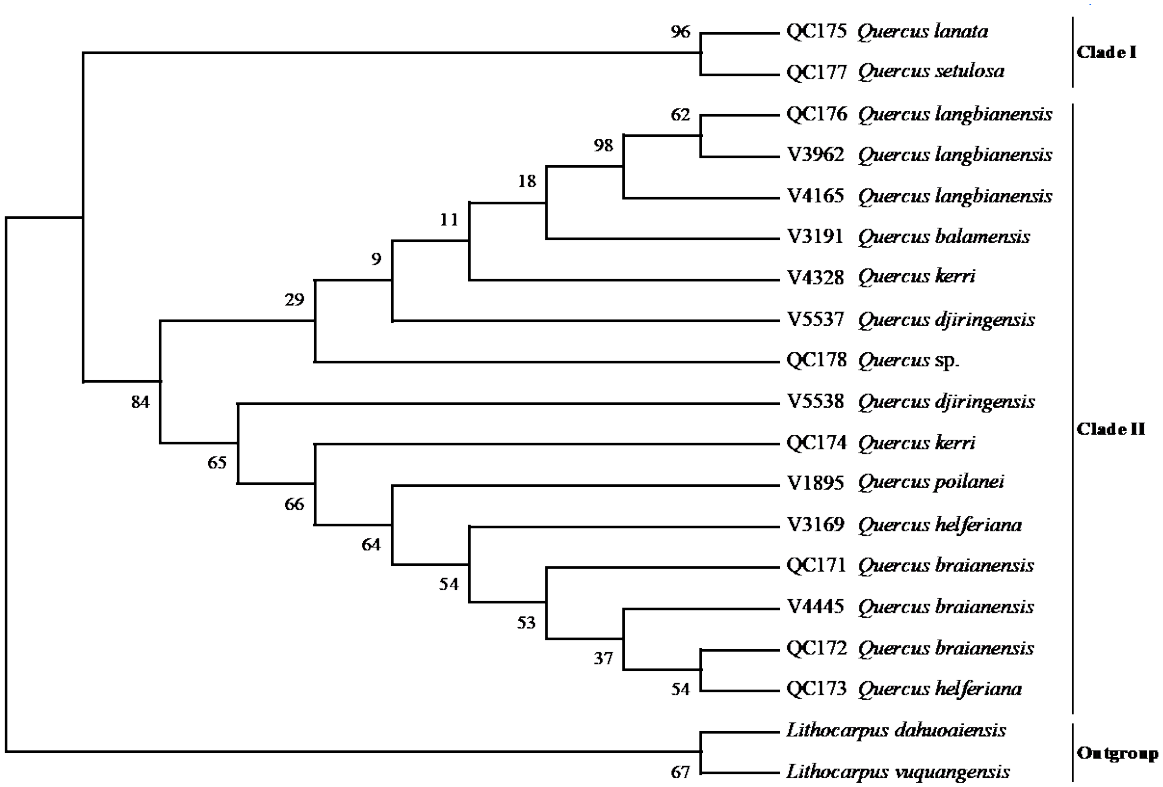

Figure 2. Neighbor-joining of *matK* sequences inferred using Kimura Two-parameter distances.

In the Bayesian tree, the *Quercus* species are also divided into two main clades (clades I and II) and are supported by the latter probabilities of 0.98 and 0.72, respectively. In particular, four species with more than one sample including *Q. helferiana*, *Q. braianensis*, *Q. djiringensis* and *Q. langbianensis* were formed into a monophyletic cluster and were supported with strong posterior probability values 0.8, 1, 0.98 and 1, respectively. Therefore, in the present study, the tree of three genes has the highest ability to distinguish *Quercus* species compared to individual loci such as ITS, *mat*K and *rbc*L.

Overall, we examined the efficacy of three barcode loci in discriminating the *Quercus* species in Lam Dong province by neighbor-joining (NJ) tree approach. According to our results, ITS and *mat*K had better discriminating *Quercus* species than *rbc*L, and the combinations of ITS, *rbc*L, and *mat*K loci were found to be the best option in discriminating the *Quercus* species. This is in line with the standard success rate of DNA barcodings for *Quercus* species discrimination level, as reported in most of the earlier studies (Piredda *et al.*, 2011; Yang *et al.*, 2017).

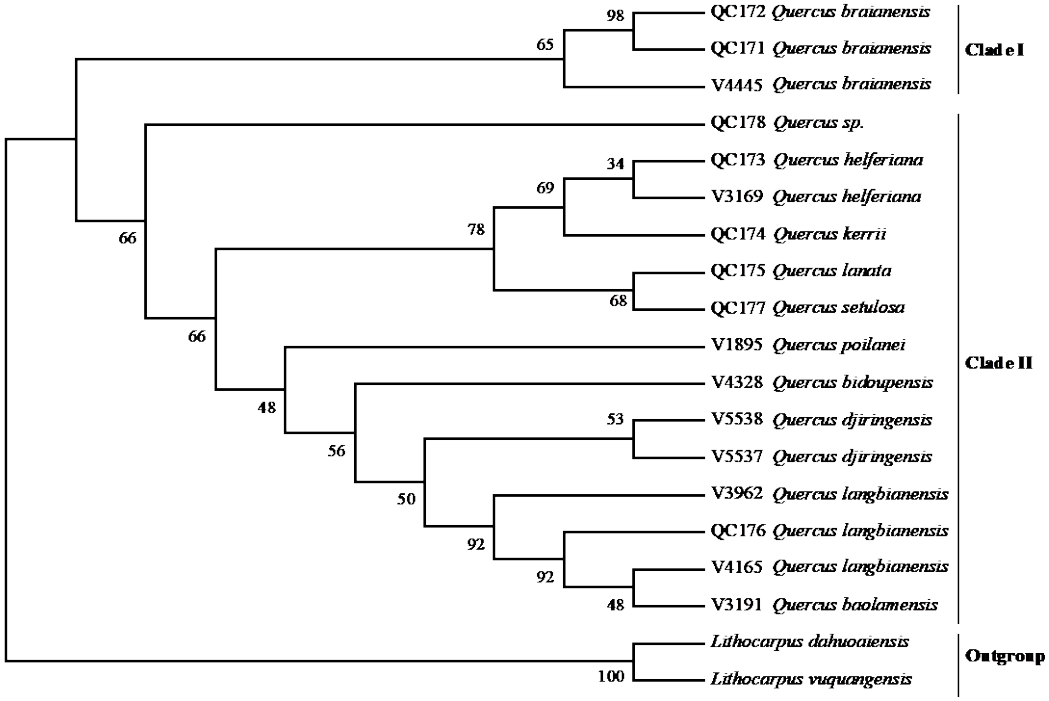

Figure 3. Neighbor joining ITS sequences inferred using Kimura Two-parameter distances of *Quercus* species in Lam Dong province and outgroup.

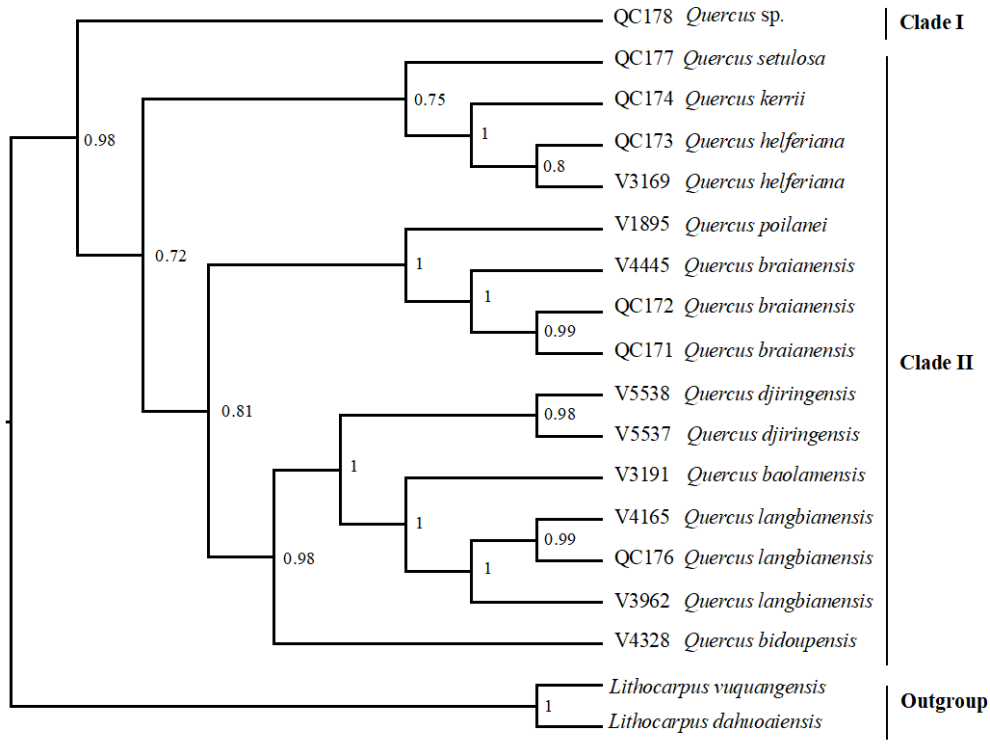

Figure 4. Bayesian phylogeny of 16 samples of *Quercus* and two samples of *Lithocarpus* (outgroup) based on *rbc*L, *mat*K and ITS sequences. Branches are labeled with posterior probabilities.

CONCLUSION

The present study evaluates DNA barcoding for discriminating the *Quercus* species distributed in Lam Dong province by BLAST-based method and tree-based method. We conclude from the present study that the rbcL, matK, and ITS markers reached the universal amplification and sequencing criteria for *Quercus* species. As a single DNA marker, the ITS region provided higher *Quercus* species discrimination than by *mat*K and *rbc*L regions. However, the combination of ITS+*mat*K+*rbc*L achieved the highest discrimination in *Quercus* species. Based on the overall performance, the combination of these three regions is proposed as the most suitable DNA barcode for identifying *Quercus* species. Also, the results of this study show that the species delimitation of *Quercus* based on molecular markers is strongly congruent with morphological based. Finally, to discriminate between *Quercus* species we can use DNA barcoding as a useful technique and provide a reliable and effective means, and in combination with morphology-based taxonomy, will be a robust approach for tackling taxonomically complex groups.

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