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DNA fingerprinting and molecular characterization of mango (*Mangifera* spp.) cultivars in Vietnam using ITS DNA barcode

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Abstract

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Mango (*Mangifera* spp.) is an important fruit tree in Vietnam. In this study, internal transcribed spacer (ITS) DNA barcode was used to characterize the genetic richness of 16 mango accessions from different provinces in Vietnam. The results show the discrimination power of ITS region. There are 14 sites of single nucleotide polymorphism detected among analyzed accessions. The dendrograms generated by cluster analysis show that the genetic similarity of samples is not strictly follow the geographical distribution of sampling sites. The obtained results provide molecular biological information for classification, identification plant origins, breeding, and conservation programs; furthermore, utilization of molecular marker analysis could provide new insights to breeders for molecular assisted selection of mango.

Keywords: DNA barcode**;** diversity; ITS; *Mangifera indica* L.; molecular markers

Introduction

Mango (*Mangifera* spp.) belongs to Anacardiaceae family which consist at least 69 species and distributed in several countries in tropical region. Morphological characteristic is mostly applied for mango classification due to its easiness to perform and carry out on the field with low cost. However; there are several limitations for this method such as low number, complex inheritance pattern and vulnerable to changes of environment (Ahmedand & Mohamed, 2014). The confused identification makes it more dificult for authenticating cultivar correctly and leads to variation in controling mango quality. For better conservation and breeding programs, several studies have focused of germplasm characterization and identification of genetic relatedness among plant accessions (Tran & Do, 2012; Ahmedand & Mohamed, 2014; Mansour et al., 2014).

To overcome the problem in morphology-based taxonomy, sequencing of genomic DNA has been served as standardized method for plant identification due to the present of homologous DNA sequences among related species (Laprise & Rodgers, 2010). Numerous studies have shown the efficiency of using DNA barcode for plant identification, it is also considered as a useful tool to study evolution of plant at molecular level (Baldwin et al., 1995; Kress et al., 2005; Hollingsworth et al., 2011; Techen et al., 2014). DNA barcode is relatively new technique which uses the standardized genomic regions to distinguish among species and has been used intensively for identifying at species-level. Mitochondrial cytochrome oxidase I (COI) gene was generally used for phylogenetic study of animals (Smith et al., 2012; Aziz et al., 2016). However; this gene is not useful in plant since lacking of sufficient variation due to its low mutation rate (Kress et al., 2005; Fazekas et al., 2009). Besides plastid DNA sequence, nuclear ribosomal internal transcribed space (ITS) region is increasingly used in plants (Chase et al., 2007; Giudicelli et al., 2015). Numerous researchers reported that ITS show higher performance in the comparison

to other plastid markers (Muellner et al., 2011; Yang et al., 2012; Zhang et al., 2014).

Mango is an important fruit tree in Vietnam and has been considered and plants for poverty production in rural areas. With total of 74 600 ha and the production of 728 100 tons/year (GSO, 2016), Vietnam stands at 13th place of mango export country (VTO, 2016). Considering the importance of mango, the study to identify the different cultivars is necessary. In spite of that, only a few studies reported genetic composition of mango in the country. Tran and Do analyzed genetic diversity of mango genotypes in Southern region by AFLP and ITS markers (Tran & Do, 2012). More recently, ISSR was utilized to exploit genetic variation of "thanh tra" (*Bouea oppositifolia* Roxb.), a distant member of mango family (Le et al., 2018). However, these two studies were only focused in Mekong delta area in the South of Vietnam. In this study, the genetic diversity of 16 mango accessions collected nationwide were analyzed by using ITS DNA barcode marker. The obtained results could provide scientific information for identification, classification and authentication of mango in Vietnam.

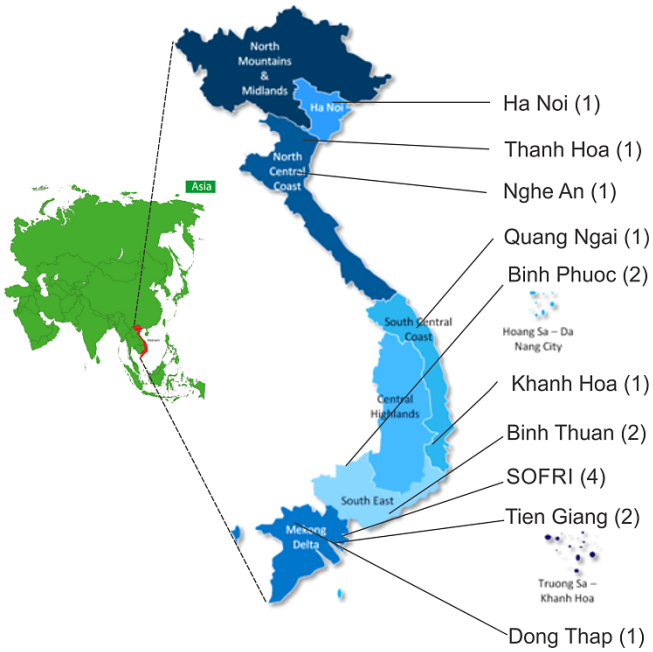

Fig. 1. Targeted areas for collecting mango genotypes (Sample number of each location is shown in parentheses)

Materials and Methods

Plant materials

Leaf samples of 16 mango genotypes were collected from germplasms of research institutes, university nurseries, and seedling centers in different provinces of Vietnam (Figure 1 and Table 1). After sampling, samples were dried in silica gel and stored until use.

DNA extraction

Total DNA was extracted from dried mango leaves using the Cetyltrimethylammonium bromide (CTAB) method described by Doyle & Doyle (1990). DNA quality was then tested by electrophoresis on 1% agarose gel in TAE 1X buffer and stained with Gelred dye (Biotium, USA). The result was observed under ultraviolet light by Quantum - ST4 3000 gel reader (Montreal - Biotech, Canada). DNA concentrations were determined by spectrophotometer (Optima SP 3000 nano UV-VIS, Japan).

ITS amplification

The ITS region was amplified using the composition of PCR reactions as follows: 7.5 µL 2X Mytaq Red Mix (Bioline, UK), 20 ng DNA, 0.2 µM primer (ITS_FW 5' ACGAATTCATGGTCCGGTGAAGTGTTCG 3' and ITS_RV 5' TAGAATTCCCCGGTTCGCTCGCCGTTAC 3') and PCR water for final volume of 15 µl. The PCR reaction conditions were as follows: initial denaturation at 95°C for 2 minutes; then 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C. Finally, addition 5 minutes was continued at 72 °C to complete the reaction. All reactions were carried out in SureCycler 8800 Thermal Cycler (Agilent, USA). The PCR products were electrophoresed using 1% agarose gel to check the presence or absence of bands. PCR amplification was then purified by ISOLATE II PCR and Gel Kit (Bioline, UK) and then sequenced using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystem, USA). The products were next run on ABI 3100 DNA analyzer (Applied Biosystem, USA). The obtained sequences were submitted to GenBank and are publicly accessible under the accession numbers listed in Table 1.

Data analysis

The obtained electropherograms were edited using FinchTV (Digital World Biology Products, USA). Finally, the sequences were blasted on NCBI BLAST under program BLASTN (National Center for Biotechnology Information, USA). Phylogenetic analysis was conducted according to the neighbor-joining (NJ), pairwise deletion for gaps/missing data, of MEGA 6.0 with 1000 bootstrap replicates. Bootstrap support (BS) was categorized as strong (> 85%), moderate (70%-85%), weak (50%-69%) or poor (< 50%) (Kress

Table 1. Mango samples collected for genetic characterization and corresponding Genband accession number

No.	Cultivar	Collected location	Sample code	Genbank accession number	Sequence length (bp)
1	Cat Hoa Loc	SOFRI	CHL-SOFRI	MN011936	650
2	Thai	SOFRI	XTH-SOFRI	MN011937	650
3	Uc	SOFRI	XU-SOFRI	MN011938	550
4	Dai Loan	SOFRI	XDL-SOFRI	MN011939	650
5	Keo	Tien Giang	XK-TG	MN011940	450
6	Cat chu	Tien Giang	XCC-TG	MN011941	650
7	Thanh ca	Dong Thap	XTC-DT	MN011942	520
8	Tu quy	Binh Thuan	XTQ-BT	MN011943	400
9	Cat trang	Binh Thuan	XCT-BT	MN011944	650
10	Queo	Quang Ngai	XQ-QN	MN011945	410
11	Rung	Binh Phuoc	XR-BP	MN011946	650
12	Tuong	Binh Phuoc	XT-BP	MN011947	600
13	Cat Hoa Loc	Khanh Hoa	CHL-KH	MN011948	650
14	Tim	Ha Noi	XTI-HN	MN011949	600
15	Cat	Thanh Hoa	XC-TH	MN011950	520
16	Tuong	Nghe An	XT-NA	MN011951	540

et al., 2002). Two ITS-cashew sequences (KF664192.1 and AB071690.1) of the family Anacardiaceae obtained from NCBI GenBank were included as out-group for phylogenetic analysis.

Results and Discussion

PCR and DNA sequence

There is a concern of the unreliability of PCR on ITS region. In order to find suitable candidate loci for DNA barcode of tropical tree species in India, Tripathi found the relatively low effectiveness of PCR in ITS region with only 74.05% success rate. In 2016, ITS marker was identified as least PCR success with only three of twelve invasive grass species in Australia (Wang et al., 2016). However, in this study, the used PCR protocol is reliable for ITS region with 100% of PCR reactions achieved. Similarly, the high success rate of PCR in ITS gene was published from different research groups. This is also in agreement to Chen and colleagues when they reported ITS is the most suitable region for DNA barcode in medical plants after surveying seven candidate DNA barcodes namely *psbA-trnH, matK, rbcL*, *rpoC1*, *ycf5*, ITS2, and ITS (Chen et al., 2010). In the comparison with other common DNA barcode markers, ITS was showed higher PCR success rate in the comparison to *matK, rbcL, ycf5* in study family Araliaceae (Liu et al., 2012). ITS region also exhibited the highest inter-specific divergence which is important for distinguishing different species. In 2016, five species in *Crawfurdia* was clearly discriminated by ITS; whereas other markers such as *trnH-psbA* and *rbcL* showed poorer results (Zhang et al., (2016).

The length of ITS sequences showed an average of 571 bp, ranging from 400 to 650 bp. The obtained sequences were then submitted to GenBank and shown in Table 1. The GC content varied from 58 to 62% with average of 60.3%. Basic Local Alignment Tool (BLAST) was used to compare sequence homology of the amplified sequences and sequences from GenBank. The sequence homology of unidentified accessions in this study is shown in Table 2, all cultivars was identified as *M. indica* with BLAST similarity from 99.17% to 100%.

The alignment of DNA sequences of 16 mango samples is showed in Table 3. There are 14 variable sites, in which there were unique sited that found specifically for each mango cultivar. MN011951 show high number of variation, the next are MN01140 and MN01148. These unique nucleotide sites can be used as diagnosis character for discriminating different mango cultivars. Such characters have been widely applied in molecular identification studies of different plant species such as *Taxus* L. (Liu et al., 2011), onion (Ipek et al., 2014), *Terminalia* sp. (Mishra et al., 2017); *Melilotus* sp. (Wu et al., 2017). By comparing several DNA barcode markers namely *rbcL*, *matK*, ITS and *psbA-trnH*, Mishra and colleagues reported that ITS marker has highest efficiency for identifying *Decalepsis* at species level (Mishra et al., 2017). A study on *Passiflora* in Brazil also reported that ITS showed higher distinguishing capacity in the comparison to either ITS1 or ITS2 alone (Giudicelli et al., 2015).

Table 2. Statistical simulation of BLAST sequence homology of unidentified specimens with ITS region

Cultivar name	Accession number	Targeting species	BLAST similarity, %	Sequence cover, %	E-value
Xoai Uc	MN011938	*Mangifera indica*	100	100	0
Xoai Dai Loan	MN011939	*Mangifera indica*	99.38	100	0
Xoai keo	MN011940	*Mangifera indica*	99.33	100	0
Xoai tim	MN011949	*Mangifera indica*	99.17	100	0

Table 3. Variable sites in 16 mango accessions based on ITS DNA barcode

Accession number	Variable sites													
	142	153	160	180	184	190	222	235	265	271	307	348	364	413
MN011936	C	T	T	C	T	T	C	C	C	G	T	C	A	C
MN011937	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MN011938	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011939	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MN011940	.	C	.	.	.	.	.	.	.	.	.	.	C	G
MN011941	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MN011942	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MN011943	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MN011944	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011945	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011946	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011947	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011948	.	C	.	T	.	.	A	.	.	.	.	T	.	.
MN011949	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011950	.	C	.	.	.	.	.	.	.	.	.	.	.	.
MN011951	T	C	C	.	C	C	.	T	T	A	G	.	.	.

Phylogenetic trees

The genetic distance among mango accessions based on the Kimura-2 parameter (K2P) is shown in Table 4. The lowest distance was 0.000, while the highest was 0.033. The low distance between sequence pairs such as MN011937 and MN111941; MN011947 and MN011949 (*M. indica*) has raised a question either individuals in these two pairs is actually the sample species, but has reported as a different species, or misidentified due to morphological complexity. This phenomenon was previously reported by Malaysian research group when using mitochondrial DNA cytochrome oxidase subunit to identify different catfish isolation (Abdullah et al., 2017). Large genetic distance between MN011951 and MN011940 suggesting that the different in genetic composition of these cultivar and may have been even two different species. Another advantages of ITS which is high evolutionary rate, high copy number of rRNA genes make its more power to discriminate at low taxonomic levels (Baldwin et al., 1995). ITS region was also supported as strong candidate for DNA barcode because it might evolve 3-4 times faster than the plastic regions and also be used successfully in studying genetic relatedness of different plants (Chase et al., 2007).

Whereas two pairs of sequences consisting of MN011946 (*M. minutiflora*) and MN011938 (*M. indica*); MN011949 (*M. indica*) và MN011945 (*M. repa*) belonging to different

Table 4. Genetic distance among 16 mango accession based on K2P method, corresponding sample codes are shown in parentheses

	Accession	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	MN011936_*M.indica* (CHL-SOFRI)															
2	MN011937_*M.indica* (XTH-SOFRI)	0.003														
3	MN011938_*M.indica* (XU-SOFRI)	0.003	0.007													
4	MN011939_*M.indica* (XDL-SOFRI)	0.003	0.003	0.007												
5	MN011940_*M.indica* (XK-TG)	0.014	0.014	0.010	0.014											
6	MN011941_*M.indica* (XCC-TG)	0.003	0.000	0.007	0.003	0.014										
7	MN011942_*M.mekongensis* (XTC-DT)	0.003	0.003	0.007	0.003	0.010	0.003									
8	MN011943_*M.indica* (XTQ-BT)	0.003	0.003	0.007	0.003	0.010	0.003	0.000								
9	MN011944_*M.indica* (XCT-BT)	0.007	0.007	0.003	0.003	0.010	0.007	0.007	0.007							
10	MN011945_*M.repa* (XQ-QN)	0.007	0.003	0.003	0.007	0.010	0.003	0.007	0.007	0.003						
11	MN011946_*M. minutifolia* (XR-BP)	0.003	0.007	0.000	0.007	0.010	0.007	0.007	0.007	0.003	0.003					
12	MN011947_*M.indica* (XT-BP)	0.007	0.003	0.003	0.007	0.010	0.003	0.007	0.007	0.003	0.000	0.003				
13	MN011948_*M.indica* (CHL-KH)	0.014	0.014	0.010	0.014	0.014	0.014	0.010	0.010	0.010	0.010	0.010	0.010			
14	MN011949_*M.indica* (XTI-HN)	0.007	0.003	0.003	0.007	0.010	0.003	0.007	0.007	0.003	0.000	0.003	0.000	0.010		
15	MN011950_*M.indica* (XC-TH)	0.007	0.007	0.003	0.007	0.007	0.007	0.003	0.003	0.003	0.003	0.003	0.003	0.007	0.003	
16	MN011951_*M.indica* (XT-NA)	0.031	0.031	0.027	0.028	0.034	0.031	0.031	0.031	0.024	0.028	0.027	0.028	0.035	0.028	0.027

species show high genetic similar. Thus, single ITS for DNA barcode could not always be perfect for mango classification, suggesting that the combination of ITS with other DNA barcode markers distributed in different genome regions could lead to more successful result in classification of mango taxonomy (Ipek et al., 2014).

The phylogenetic tree constructed by using MEGA 6.0 with the neighbor-joining (NJ) method is shown in Figure 2. The bootstrap value showing the separation support of the in-group and out-group is up to 99%. Two ITS sequences of cashew obtained from Genbank (accession number KF664192.1 and AB07.1690.1) formed a clear and observable clade to other mango accessions indicating the reliability of phylogenetic result. The studied mango accessions were divided into two main groups based on NJ analysis. Only accessions MN011951 collected from Nghe An is separated from others. Generally, studied accessions are distributed in groups responding to geographical locations. Though, some accessions are grouped together but they were collected in different distant locations such as MN011949, MN011945 and NM011947 collected from North (Hanoi), Central region (Quang Nam), and South region (Binh Phuoc), respectively. This result suggests there is the mixture among accessions.

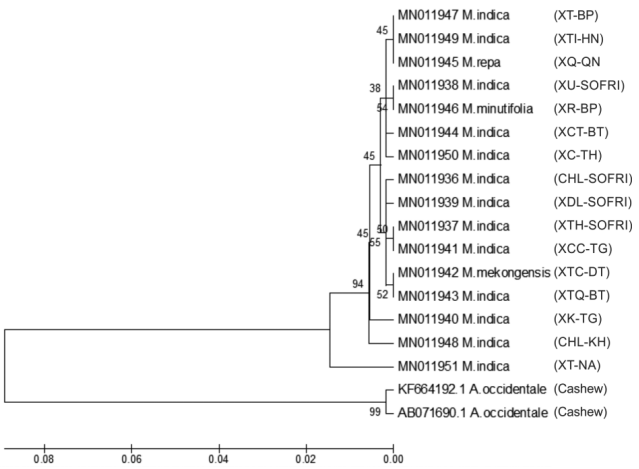

Fig. 2. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Kimura 2-parameter method. Letters in parentheses indicating the sample codes, the scale bar showing the units of the number of base substitutions per site

Conclusions

DNA barcoding has been widely chosen for authenticating materials from herbal plants, product substitution and contamination. Our results show that ITS marker is effective to identify mango accessions. The advantages of DNA barcoding which could make the identification of plant species become easier for non-experts in molecular biology because time and labor could be reduce significantly in the comparison to morphological identification. The obtained results in this study prove that the use of ITS DNA barcode is successful, for amplification, identification and discrimination at species level of mango. These findings would be potentially for genetic characterization and classification in plant genetic resource management and breeding.

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