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Identification of Mango (*Mangifera indica* L.) cultivars in the Mekong Delta using ISSR markers and DNA barcodes

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ABSTRACT

Mango (*Mangifera indica* L.) is a biologically and economically important fruit crop in Vietnam. However, the authentication of various cultivars and accessions of *Mangifera indica* L. has still been limited. In this study, 10 Inter simple sequence repeats (ISSRs) molecular markers and four DNA barcodes (ITS, *ycf1b*, *trn*H-*psb*A, and *atp*F-*atp*H) were used to distinguish 30 accessions of three mango cultivars (Chau Nghe, Hoa Loc, and Cat Chu). DNA was extracted using the cetyltrimethylammonium bromide protocol, and the internal transcribed spacer (ITS), ycf1b, trnH-psbA, and atpF-atpH regions were amplified and sequenced for alignment analysis. Based on ISSR data, 76 bands were generated, with the percentage of polymorphism equal to 61.84%. The phylogenetic tree constructed from ISSR data showed significant genetic variation between Chau Nghe and the other mango cultivars. In terms of barcoding assessment, nuclear region ITS and plastid gene *ycf1b* only discriminated between mango cultivars while two remaining plastid locus could be used for authentication of the difference of mango accessions. The barcoding results also confirmed close genetic relatedness between Mekong Delta mango cultivars and other species of the Mangifera genus from the National Center for Biotechnology Information database. These findings provide new insights into mango cultivar identification, classification, breeding, and conservation.

1. INTRODUCTION

Mango (*Mangifera indica* L.) belonging to the family Anacardiaceae, with the botanical classification Mangifera genus, producing a drupe-type fruit with a panicle inflorescence is one of the most important crops grown in tropical regions [1]. According to the Ministry of Agriculture and Rural Development of Vietnam, ranks 13th globally in mango production. Mekong Delta is the largest mango cultivation area, accounting for 48% of the 87,000 hectares nationwide with many cultivars including Hoa Loc, Cat Chu, Chau Nghe, Thai, and Taiwan [2]. Additionally, mangoes are packed with essential nutritional elements like vitamin C, vitamin A, potassium, and dietary fiber, along with powerful phytochemicals such as mangiferin, beta-carotene, polyphenols, and flavonoids, all contributing to their health-promoting benefits. The appropriateness of climate and alluvial soil make a contribution to the abundance of different cultivars belonging to *M. indica* L. in the Mekong Delta. However, reports that shed light on the authentication of mango cultivars and a clear trademark system for them have been limited. Therefore, it is an urgent demand in the assessment of cultivar identity, genetically diverse level, and parental selection for the breeding program of *M. indica* L. in Vietnam [3].

Molecular assessment is superior to morphological and chemical characterization as DNA-based markers are stable and detectable in all tissues, regardless of any developmental stage of the cell, from growth to differentiation or state of defense. Another advantage of molecular markers is that they are not affected by environmental, pleiotropic, or epistatic factors [4]. Inter simple sequence repeats (ISSRs) are based on regions between adjacent, oppositely oriented microsatellites. They are non-specific markers so no requirement for sequence information for primer construction is needed [5]. For genetic identification, ISSRs were informative in categorizing Thai mango accessions when over 80% of the bands were polymorphic among the 78 bands generated [6]. ISSR markers revealed high polymorphism among mango cultivars from ten provinces in Vietnam [7].

DNA barcoding is a leading-edge molecular system that utilizes different short, standardized DNA fragments for species authentication. Thus, DNA barcoding has been proposed as a prospective candidate for the evaluation of species identity, the evolution of ecological populations, protective status, and biodiversity [8]. In terrestrial plants, the plastid genome contains two core barcodes for DNA barcoding: rbcL and matK, while intergenic regions within the chloroplast (*trn*H-*psb*A) and nuclear genome internal transcribed spacer (ITS) would serve as the accompanying

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sources for the construction of DNA barcoding database [9]. *rbc*Lmainly encodes for the key photosynthesis enzyme ribulose bisphosphate carboxylase (RuBisCo), while *mat*K codes for a mature enzyme that is responsible for type II intron splicing throughout RNA transcripts [10]. *trn*H-*psb*A is considered an efficient tool in species discrimination owing to its genetic variability. However, it is uncomplicated to design *trn*H-*psb*A as a universal primer due to the 75-bp conserved regions at two ends of this intergenic region [11]. The internal transcribed spacer (ITS) regions are non-coding, highly variable sequences surrounding the 5.8S ribosomal RNA gene. In the plastid genome, *ycf1b* is one of two regions belonging to gene *ycf1* that are necessary for plant viability [12]. In addition, two non-coding plastid regions (*atp*F–*atp*H and *psb*K–*psb*I) are commonly combined with two core barcodes in species identification and phylogenetic construction. Multi-locus DNA barcode was applied to identify different taxa of a desert plant called *Rhazya stricta*, in which the plastid spacers *psb*K-*psb*I and *atp*F-*atp*H revealed highly diverse among tested taxa compared to that of coding regions *mat*K, *rbc*L [13]. Regions of the plastid genome (*rbc*L, *mat*K, and *trn*L-F) and nuclear genome (ITS) were assigned as DNA barcodes to authenticate 14 grass accessions [14].

Both DNA-based markers and DNA barcodes are virtually exempt from the stage of development, physiological state, and environmental factors, thereby being used as quick, reliable techniques in species identification, phylogenetic construction, and genetic relationships.

Table 1. List of ISSR markers.

No.	Sequence (5’-3’)	Tm (ºC)
1	GAGAGAGAGAGAGAGAC	52.4
2	CACACACACACACACAG	52.4
3	ACACACACACACACACT	50.0
4	ACACACACACACACACC	52.4
5	ACACACACACACACACG	52.4
6	AGAGAGAGAGAGAGAGG	52.4
7	GAGAGAGAGAGAGAGACT	53.8
8	ACACACACACACACACCT	53.8
9	ACACACACACACACACCA	53.8
10	ATGCACACACACACACA	50.0

Therefore, this study utilized ten ISSR markers to evaluate the genetic relatedness of three mango cultivars (Chau Nghe, Hoa Loc, and Cat Chu). Additionally, we created a barcode database using regions from the nuclear genome (ITS) and plastid genome (*ycf1b*, *trn*H-*psb*A, and *atp*F-*atp*H) and assessed the genetic relationship among different accessions of observed mango cultivars.

2. MATERIALS AND METHODS

2.1. Materials

30 leaf-samples of Chau Nghe, Hoa Loc, and Cat Chu cultivars were collected from Tra Vinh province. Each cultivar includes ten samples. The letters N, L, and C were used to code the Chau Nghe, Hoa Loc, and Cat Chu accessions, respectively, followed by a number from 1 to 10. Chau Nghe mango was collected from Dua Do 3 hamlet, Nhi Long Phu commune, Cang Long district. Hoa Loc mango was collected from Soc Moi hamlet, Long Son commune, Cau Ngang district. The first five Cat Chu accessions (from C1 to C5) were collected from An Loc hamlet, Hoa Tan commune, Cau Ke district while the remaining accessions were from Tan Qui II hamlet, An Phu Tan commune, Cau Ke district.

Chemicals for DNA extraction: Liquid nitrogen, CTAB Buffer, TE 1X (Merck, USA), TE 0.1X, Isopropanol, Chloroform (made from Chloroform and Isoamyl alcohol (24:1)), ethanol absolute 95%, ethanol (70%). PCR and DNA electrophoresis: BiH_20, PCR Buffer (a composition of dNTPs, Tag polymerase, $MgCl_2$, buffer solution); forward and reverse primers; pure agarose; dyes of Safeview, TBE 1X diluted from TBE 50X (Bio-rad, USA), loading buffer, ladder (GeneRuler 100 bp).

2.2. Methods

2.2.1. DNA extraction

The extraction of DNA from dried mango leaves was performed according to the cetyltrimethylammonium bromide (CTAB) method described by Rogers and Bendich [15]. The DNA samples were stored at −20°C until use. DNA quality was evaluated using electrophoresis on 1% agarose gel in TBE 1X buffer, stained with SafeView. The result was observed under ultraviolet light by Bio-rad UV2000. DNA concentrations were determined by Nanodrop One (America).

Table 2. List of DNA barcoding markers and PCR cycling for DNA barcoding sequences.

Primer	Sequence (5’-3’)	Initial denaturation	35 cycles			Final extension
			Denaturation	Annealing	Extension	
*atp*F-*atp*H	*atp*F: ACTCGCACACACTCCCTTTCC	94ºC	94ºC	51ºC	72ºC	72ºC
	*atp*H: GCTTTTATGGAAGCTTTAACAAT	4 minutes	30 seconds	40 seconds	40 seconds	5 minutes
*trn*H-*psb*A	*psb*A3F: GTTATGCATGAACGTAATGCTC	94ºC	94ºC	55ºC	72ºC	72ºC
	*trn*HF05R: CGCGCATGGTGGATTCACAATCC	4 minutes	30 seconds	30 seconds	1 minutes	10 minutes
ITS	*ITS*1: TCCGGGAACCTGCGG	95ºC	95ºC	56ºC	72ºC	72ºC
	*ITS*4: TCCTCCGCTTTGATGC	5 minutes	30 seconds	30 seconds	1 minutes	5 minutes
ycf1b	*ycf1b*F: TCTCGACGAAAATCAGATTGTTGTGAAT	94ºC	94ºC	59ºC	72ºC	72ºC
	*ycf1b*R: ATACATGTCAAGTGATGGAAAA	4 minutes	30 seconds	40 seconds	1 minutes	10 minutes

(Source: Primer database from boldsystems.org)

2.2.2. ISSR amplification and analysis

The nucleotide sequences of ten ISSR primers used [16-17] are described in Table 1. The components of PCR consisted of 10 µl 2X Master Mix, 50 ng DNA, 20 pmol/µl primer, and ultrapure water for a final volume of 25 µl. The thermal cycling conditions of ISSR procedure were conducted as follows: 4 minutes for initial denaturation at 94°C; then 35 cycles of 1 minute at 94°C, 45 seconds at 50°C, and 2 minutes at 72°C and 7 minutes for final extension at 72°C. The PCR procedure was performed with MultiGene™ OptiMax Thermal Cyclers (USA).

Next, the quality of PCR products was evaluated by electrophoresis on 2% agarose gel in TBE 1X buffer and stained with SafeView. The result was detected under ultraviolet light by Bio-rad UV2000. The presence or absence of bands was recorded in binary code as 1 or 0, respectively. Matrices of similarity and pairwaise distance were analyzed, thereby creating a dendrogram according to cluster analysis by using NTSYSpc 2.1 program based on character differences. The software tool iMEC was used to determine the Polymorphic Information Content (PIC) [18].

2.2.3. DNA barcode amplification and analysis

Sequences of the primers for mango barcoding evaluation were sourced from boldsystems.org, as indicated in Table 2. The PCR composition for DNA barcoding amplification included 20 µl of Master Mix, 50 ng of DNA, 1 µl of forward primer, and 1 µl of reverse primer. Ultrapure water was added to achieve a final volume of 50 µl. The thermal cycling conditions for the PCR procedure are shown in Table 2.

PCR products that exhibited clear bands on a 2% agarose gel were sent to a DNA sequencing company. Tax code: 1801742446. Address: U34C Six street, Hung Phu residential area, Hung Thanh ward, Cai Rang district, Can Tho city, Vietnam.

The sequences and compositions of DNA fragments for each mango genotype were analyzed using BioEdit version 7.0.5.3 software. The selection of consensus sequences (common ones) was based on the alignment of DNA segments within the same species. Afterward, the variable sites were determined by nucleotide comparison of these consensus sequences with one another on the MEGA-11 program.

4. RESULTS

4.1. ISSR Amplification and Sequencing Analysis

The data obtained (Table 3) indicated that a total of 76 bands were generated, of which 47 (61.84%) showed polymorphism.

The size of amplified bands was between 200 and 1500 base pairs (bps). The polymorphic percentage ranged from 17% (marker UBC809) to 100% (marker ISSR827) with an average of 61.9%. The PIC value ranged from 0.32 (ISSR826 and ISSR827) to 0.37 (UBC840, UBC855, and UBC888). The resolving power (RP) value varied from 4.3 (marker UBC809) to 16.3 (marker ISSR827). These data illustrated that ISSR markers used are suitable for amplifying regions with highly genetic variation between mango cultivars and accessions.

Markers ISSR811, ISSR818, and ISSR818 generated unique bands for discrimination between different accessions of the same cultivars as well as between different cultivars In contrast, remaining primer pairs used just could be used for cultivar characterization especially between Chau Nghe mango and two other mango cultivars when each marker gave at least one fragment that only appears in Chau Nghe accessions.

Among all the mango samples observed, two accessions, namely L1 and L3, did not amplify with all ISSR markers used.

Based on the Unweighted Pair Group Method with Arithmetic Mean cluster analysis (Fig. 1), 28 mango cultivars and accessions were separated into two major clusters composed of four groups with a similarity coefficient of 0.44. First, 9 out of 10 Chau Nghe mango accessions belonged to the same group. Among these, accessions N5 and N6 exhibited the highest similarity coefficient of 100%. At a cut-off value of 0.89, accession N2 was separated from the remaining Chau Nghe samples. It means that N2 is genetically most distantly related to other Chau Nghe accessions observed. Group 3 consisted of five accessions from Loc mango (L2, L5, L7, L8, and L9) and four accessions from Cat Chu mango (C1, C6, C7, and C9) with a similarity coefficient of 0.64. Regarding group 4, C5 had the highest coefficient with C8 (0.99), while C3 illustrated the farthest genetic distance compared with other samples of the same group with a similarity coefficient of 0.8.

The result of gel electrophoresis showed that ITS, ycf1b, trnH-psbA, and atpF-atpH have fragment lengths of 700 bp, 800 bp, and 500 bp for the two latter, respectively, which was equal to the expected band sizes. Figure 2 shows that most band patterns of mango genotypes with trnH-psbA were clearly visible on a 2% agarose gel. There was a missing band at N2 genotype and smeared DNA bands at N5 and N7 samples. The missing band of mango samples could be due to the variation of mango genotypes, thereby unsuccessfully amplifying these genotypes with barcoding markers. Among 30 mango samples used, *trn*H-*psb*A and *atp*F-*atp*H (50%) had the highest success rate of PCR amplification, while the success rate of PCR amplification

Table 3. Description for amplified result of ISSR marker.

Marker	Total bands	Polymorphic fragments	Polymorphic percentage (%)	Fragment size range (bp)	PIC	RP
ISSR811	6	4	67	400–1,500	0.35	15.7
ISSR818	7	5	71	350–1,500	0.33	14.0
ISSR825	6	5	83	200–900	0.36	16.0
ISSR826	8	7	88	400–1,200	0.32	11.8
ISSR827	8	8	100	350–1,200	0.32	16.3
UBC809	6	1	17	200–700	0.36	4.3
UBC840	10	6	60	200–1,500	0.37	7.4
UBC855	6	3	50	400–1,500	0.37	13.0
UBC856	11	5	45	200–1,500	0.35	10.9
UBC888	8	3	38	200–900	0.37	8.8

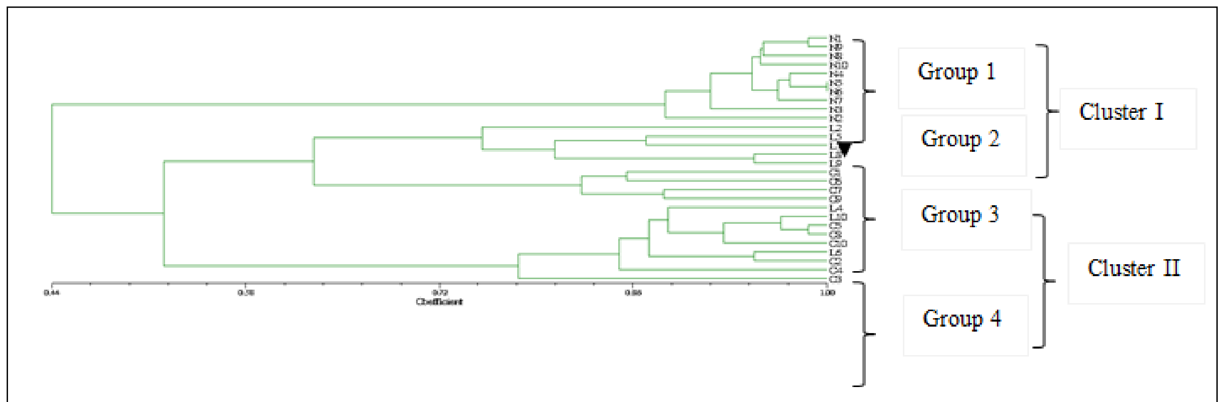

Figure 1. Dendrogram of the ISSR markers for 28 mango genotypes.

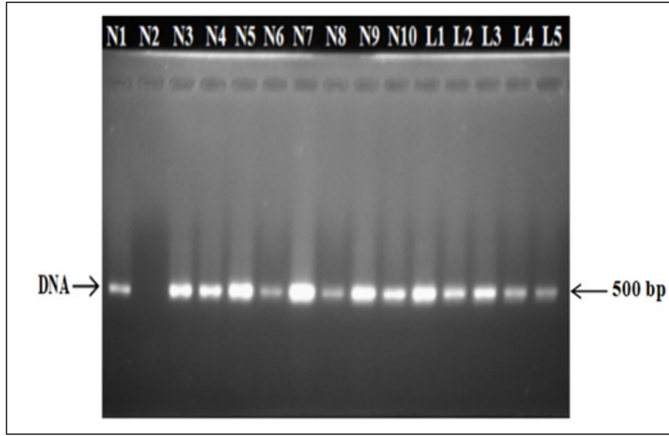

Figure 2. PCR products with trnH-psbA primer on agarose gel.

for ITS was the lowest (43.33%). For DNA sequencing, *atp*F-*atp*H demonstrated the highest success rate at 50%, followed by ITS at 26.67%, and *trn*H-*psb*A at 23.33%.

The alignment results indicated that all observed mango genotypes displayed a high level of genetic conservation (Table 4). As a result, *M. indica* L. cultivars indicated their close genetic relatedness. However, the genetetic diversity of the studied mango fruit cultivars and accessions was evidenced by the presence of SNPs and indel mutations. The nuclear ITS marker exhibited numerically superior variability compared to other spacers used with 78 variable sites. However, *trn*H-*psb*A and *atp*F-*atp*H markers occupied the largest parsimony informative sites among the variable sites detected. Regarding indel mutations, *trn*H-*psb*A revealed their diversity among mango samples used, followed by ITS and *ycf1b*, with six and three mutations detected, respectively.

Regarding the ITS spacer, the difference between N3, N7, and other samples was observed due to an indel mutation of the C nucleotide and a SNP (A/G), respectively (Fig. 3). Moreover, N1 was distinguished from other mango samples by an indel mutation of G nucleotide and a SNP as C/G. The discrimination between Chau Nghe accessions and a Loc mango accession (L7) was revealed by 76 SNPs and an indel mutation of C nucleotide at locus 646. Regarding *ycf1b*, accession N9 differed from other mango samples by a SNP (T/G), at loci 195 and 199. The difference of L5 was successfully identified by an indel mutation of T nucleotide and five substitution mutations, including A/G, T/G, G/A, and T/A (Fig. 4). The divergence of L10 from other mango samples was detected through 23 SNPs and two indel mutations of A nucleotide at loci 328 and 365. From the alignment result, it could be concluded that *ycf1b* is an effective locus for discrimination between Chau Nghe mango and some accessions of Hoa Loc mango. In contrast, the divergence between Chau Nghe and Cat Chu mango could not be distinguished using this barcoding gene.

In terms of *trn*H-*psb*A, serial positions from 8 to 14 and 16–21 were the most informative variable sites for discrimination between mango cultivars (Chau Nghe and Cat Chu mango) and Chau Nghe accessions (Fig. 5). Six singleton sites (14, 21, 42, 47, 51, and 52) and an insertion of nucleotides at loci 27 and 31 were detected, contributing to the identification of accession N1 from other mango samples. The difference between accession C1 and other mango samples was identified through sequences of SNPs, namely TCTTAA and ATTACAAA. In addition, a serial deletion of five nucleotides AAAAA or AAATA was found on a Cat accession (C6), thereby distinguishing it from other mango samples observed.

From the alignment results, *atp*F-*atp*H was considered the most informative and effective marker for discriminating between mango cultivars and accessions belonging to *Mangifera indica* L. in Vietnam (Fig. 6). Accession N10 was distinguished from other mango samples by an indel mutation of G nucleotide and SNPs substitution mutations, including A/G, C/A, A/T, and G/T. Five Chau Nghe samples (N5, N6, N8, N9, and N10) exhibited a close relationship with a Loc accession (L2) based on G and A nucleotides at four loci (259, 261, 266, and 268). N1 was clearly different from other mango samples through substitution mutations, including A/T, T/C, G/T, G/A, and G/C.

Using standard nucleotide BLAST from NCBI, it was founded that four mango genotypes from India, *M. indica* (MF444902.1, OL960664.1, and AB598049.1), *Mangifera odorata* (MF444901.1) showed the identity percentage from 98.68% to 99.55% compared with *Mangifera indica* L. tested with nuclear gene fragment ITS in our study (Table 5). Similarly, *Mangifera indica* L. samples tested with other DNA barcoding genes revealed the identity percentage from 96.25% to 100% compared with the following accessions including *M. indica* (MN711724.1, KX871231.1, NC_035239.1) and *M. sylvatica* (MN786795.1) from three different countries. The high identity percentage demonstrated genetic relatedness between three cultivars of *M. indica* L., with those accessions available on NCBI.

Table 4. Characteristics of aligned sequences of four DNA barcode candidates.

Locus	Aligned length (bp)	Conserved sites	Variable sites		Indel mutations
			Singleton sites	Parsimony informative sites	
ITS	683	604	78 (11.4%)	0 (0%)	3 (0.44%)
ycf1b	786	754	28 (3.56%)	1 (0.13%)	3 (0.38%)
*trn*H-*psb*A	346	312	21 (6.07%)	12 (3.47%)	6 (1.73%)
*atp*F-*atp*H	378	352	13 (3.44%)	12 (3.17%)	1 (0.26%)

Samples	Positions																																							
	2	6	10	58	426	429	430	433	438	445	447	451	455	460	462	464	470	472	475	476	482	484	486	490	498	500	504	507	509	512	515	517	523	525	532	535	538	542	549	554	556
N1	C	A	G	G	G	C	T	C	C	C	T	T	C	A	T	G	C	T	G	C	C	T	C	T	A	C	G	C	C	T	C	C	C	C	C	T	C	T	C	T	G
N2	.	.	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N3	-	.	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N4	.	.	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N5	.	.	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N6	.	.	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N7	.	G	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L7	.	.	-	C	A	T	A	G	T	G	G	G	G	T	G	C	G	G	C	T	G	G	G	G	T	T	T	T	G	G	G	T	G	G	G	G	G	G	A	A	T

Samples	Positions																																						
	557	562	563	566	568	570	574	576	580	584	591	593	594	596	598	600	607	609	612	615	624	626	630	631	633	635	646	648	656	658	662	664	665	666	670	672	676	677	678	679
N1	T	C	T	C	T	C	T	C	C	C	G	G	C	C	T	G	G	G	C	G	C	C	C	A	C	C	C	G	C	C	C	G	A	G	A	G	A	T	C	A
N2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L7	G	T	G	G	G	G	G	G	T	G	A	C	T	T	C	A	A	A	G	C	T	T	A	T	G	G	-	A	T	A	G	T	G	A	T	A	T	A	T	C

Figure 3. Variable sites and indel mutations of nuclear fragment ITS.

Samples	Positions																														
	195	199	245	250	253	282	289	291	295	296	298	299	300	301	302	303	304	305	306	307	311	312	318	319	320	321	324	328	348	355	359	365
N2	T	T	T	A	-	T	T	G	T	A	A	G	A	A	C	G	T	A	C	T	C	T	C	T	C	T	A	-	T	T	T	-
N3	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N4	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N5	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N6	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N7	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N8	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N9	G	G	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
N10	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
L2	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
L5	.	.	.	G	T	G	G	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
L7	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
L9	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-
L10	.	.	A	.	-	.	.	.	A	T	G	A	G	C	G	C	G	T	A	C	T	C	T	C	T	C	G	A	G	G	G	A
C7	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	-

Figure 4. Variable sites and indel mutations of *ycf1b* gene.

Samples	Positions																											
	2	4	5	7	8	9	10	11	12	13	14	16	17	18	19	20	21	22	23	25	26	27	28	29	31	42	47	51	52
N1	C	G	G	C	C	T	C	C	A	A	C	A	C	T	C	A	C	A	C	A	A	A	A	A	G	C	C	T	A
N3	.	.	C	.	T	.	.	.	T	T	A	.	A	.	T	.	A	C	A	.	.	.	T	.	-	A	T	A	T
N4	.	.	C	.	T	.	.	.	T	T	A	.	A	.	T	.	A	C	A	.	.	-	T	.	-	A	T	A	T
N8	.	.	C	.	T	.	.	.	T	T	A	.	A	A	T	T	A	.	.	.	.	-	T	.	-	A	T	A	T
N10	G	.	.	G	.	.	.	T	T	.	A	.	A	.	T	.	A	C	.	.	.	-	T	.	-	A	T	A	T
C1	.	.	C	.	T	C	T	T	.	.	A	.	T	.	A	C	A	.	A	.	.	-	T	.	-	A	T	A	T
C6	.	C	A	T	.	.	T	A	.	.	A	T	T	A	.	.	A	.	A	-	-	-	-	-	-	A	T	A	T

Figure 5. Variable sites and indel mutations of *trn*H-*psb*A spacer.

5. DISCUSSION

Overall, the percentage of polymorphic bands reported here was higher than those described in previous studies on mango fruit by Kheshin *et al.* [19], Hidayat *et al.* [20], and Ghounim *et al.* [21], but lower than the figures reported by Ganogpichayagrai *et al.* [6] and Ho and Tu [7].

All of the markers used in this study showed a moderate PIC value, meaning that ISSR markers are appropriate for assessing genetic

Samples	Positions																								
	16	17	247	255	259	261	266	268	271	284	286	300	310	313	317	321	326	334	336	340	348	352	344	366	370	374
N1	G	-	T	C	T	C	T	A	T	A	A	C	A	G	A	T	C	T	C	A	A	A	A	A	A	G
N3	.	-	A	T	G	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	T	.
N4	.	-	A	.	.	G	.	.	.	.	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.
N5	.	-	A	.	G	G	A	G	.	.	.	.	G	.	.	A	.	A	.	.	.	.	.	.	.	.
N6	.	-	A	.	G	G	A	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.
N7	.	-	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N8	.	-	A	T	G	G	A	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	G	T	.
N9	.	-	A	T	G	G	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N10	T	G	.	.	G	G	A	.	.	T	T	A	.	.	G	.	.	.	.	T	T	T	G	.	.	.
L2	.	-	A	.	G	G	A	G	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L5	.	-	.	T	G	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
L7	.	T	A	.	G	.	A	G	.	.	.	.	G	.	.	.	.	.	G	.	.	.	.	G	.	.
L8	.	-	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
L9	.	-	A	.	G	.	.	.	.	T	T	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.
C7	.	-	A	.	G	.	.	.	.	T	T	.	.	T	.	.	A	.	.	.	.	.	.	.	T	C

Figure 6. Variable sites and indel mutations of *atp*F- *atp*H spacer.

Table 5. Nucleotide polymorphism between *M. indica* L. and other *Mangifera* genotypes on NCBI database.

Locus	Length (nt)	Coverage (%)	Identities (%)	Number of gaps	Accessions
ITS	683	97%–100%	98.68%–99.55%	0	*M. indica* (MF444902.1)
					M. indica (AB598049.1)
					M. odorata (MF444901.1)
					M. indica (OL960664.1)
trnH-psbA	346	100%	96.25%	0	*M. indica* (MN711724.1)
					M. sylvatica (MN786795.1)
atpF-atpH	378	100%	98.94%	1	*M. indica* (KX871231.1)
ycf1b	786	100%	100%	3	*M. indica* (NC_035239.1)

relatedness in mango fruit, according to Botstein *et al.* [22]. In addition, the RP index reflects the discriminatory proficiency of a primer to differentiate a genotype or individual [23].

The result of ISSR analysis is consistent with the findings reported by Ho and Tu [7], in which Hoa Loc mango and Cat Chu mango belonged to two different groups of the same cluster. The phylogenetic result indicated that the studied mango accessions from Hoa Loc and Cat Chu cultivars were not classified as geographical distributions where samples were accumulated. This may be attributed to the exchange of accessions among local mango cultivation areas. In addition, hybridization between closely related genotypes among *Mangifera indica* accessions in Brazil was demonstrated by the high level of endogamy with an index value of 0.6 [24].

5.1. DNA Barcoding Analysis

The success rate for both PCR amplification and sequencing is consistent with that reported by Kang *et al.* [10] when using four DNA barcodes (*rbc*L, *mat*K, *trn*H-*psb*A, and ITS) for identification of the genetic relatedness of species in tropical cloud forests.

According to Ashour *et al.* [25], the nuclear spacer (ITS) is located between the 18S rRNA and 28S rRNA genes, which are arranged in tandem repeats. In eukaryotic species, rRNA genes are considered an efficient tool for phylogenetic assessment owing to their rapid evolution and effortlessness in amplification and sequencing. Although the nuclear spacer (ITS) did not successfully distinguish the genetic variation between Hoa Loc and Cat Chu mangos cultivated in Vietnam reported by Do *et al.* [26] and Ho *et al.* [27], this barcode region showed their high polymorphism for discriminating between Chau Nghe mango and Hoa Loc mango in this study. In addition, the genetic distance matrix of *M. indica* L. was from 0 to 0.1118, with an average of 0.0286. Sequencing the ITS regions between *Mangifera indica* L. landraces collected from south Iran indicated that the genetic distance matrix ranged from 0.02551 to 0.47224 [28]. Although mango landraces cannot be clearly distinguished by ITS, two Manojan and Jroft landraces were categorized as different groups.

The gene *ycf1b* was demonstrated as a promising DNA barcode for identifying precisely different species belonging to Zingiberaceae, in which the interspecific difference showed a superior mean to that of the intraspecific one [29]. With a size of around 1kb, ycf1b has been acknowledged as a significantly efficient plastid DNA barcode for species identification, especially in terrestrial plants [12]. Two out of seven species belonging to *Pinus* could not be clearly distinguished by phylogenetic analysis by *ycf1b* [30]. These results are consistent with our study, where discrimination between Chau Nghe mango and Cat Chu mango was not successfully identified. Barcode *trn*H-*psb*A is proposed as the most genetically diverse plastid spacer and has the ease to amplify across a large assortment of terrestrial plants [31]. According to Feng *et al.* [32], markers from the intergenic region *trn*H-*psb*A exhibited their efficiency in identifying the interspecific

and intraspecific polymorphisms of *Physalis*. By using the chloroplast marker *trn*H-*psb*A, the molecular diversity of 14 genotypes of *M. indica* L. cultivated in India was indicated [33]. Moreover, a clear identification between sweet and bitter almonds was indicated by using the coding plastid region *trn*H-*psb*A [34]. According to Thakur *et al.* [35], intergenic regions *atp*F-*atp*H and *trn*H-*psb*A were successfully identified not only for the polymorphism between 28 plant families in India but also for molecular diversity between species of the same family.

Genetic variation of intraspecies could arise from multiple cross-hybridizations among several species [36]. According to Muthukumar *et al.* [37], the variation of different cultivars belonging *M. indica* L. due to their evolutionary processes including natural selection and open pollination. This was confirmed by the sequence distinction in chloroplast genes (*trn*L and *trn*F) among eight different genotypes of *M. indica* L. in India. Their findings also proved that the inheritance of chloroplast genome in mango was not strictly maternal but could be bi-parental or paternal owing to the heterozygous and heterogeneous nature of the species.

6. CONCLUSION

The biological significance of ISSR markers for studying the genetic diversity and genetic relationship among three *M. indica* L. cultivars (Chau Nghe, Hoa Loc, and Cat Chu) was determined by the obtained data. A phylogenetic tree constructed by ISSR markers indicated that Chau Nghe mango has distant genetic relatedness with other cultivars of *Mangifera indica* L. In addition, markers ITS and *ycf1b* successfully discriminated between different cultivars of mango fruit, while other plastid DNA barcode regions (*trn*H-*psb*A and *atp*F-*atp*H) clearly and effectively identified mango accessions. This study demonstrated that molecular markers and DNA barcode sequences are efficient tools for detection and discrimination at the species level of mango fruit. These findings are an indispensable breakthrough for the assessment of genetic characteristics and sub-species classification in the conservation and breeding of Vietnam's mango germplasm.

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8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines

9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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