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GENETIC CHARACTERIZATION OF MANGO ACCESSIONS THROUGH RAPD AND ISSR MARKERS IN VIETNAM

VIET THE HO[1,*] and NGAN TRAM TU[2]

[1]Ho Chi Minh City University of Food Industry, 140 Le Trong Tan Street, Tan Phu District,
Ho Chi Minh City, Vietnam
[2]Ho Chi Minh City University of Technology, 475A Dien Bien Phu Street, Binh Thanh District,
Ho Chi Minh City, Vietnam
*Corresponding author's email: thehv@hufi.edu.vn
Email address of coauthor: tungantram@gmail.com

SUMMARY

Molecular markers have been used to study the genetic diversity in mango (*Mangifera indica* L.) through characterization worldwide. In this study, 10 RAPD and 10 ISSR markers were used to characterize the genetic richness of 10 mango accessions from different provinces in Vietnam. High polymorphic levels were found in both RAPD (91.9%) and ISSR (98.1%) markers in which a total of 162 and 145 loci were generated, respectively. Both markers revealed high polymorphism information content (PIC) (0.89 for RAPD and 0.91 for ISSR), suggesting that these methods are equally effective for determining genetic variation of mango. However, the Mantel test showed no correlation between these two markers. The dendrograms generated by cluster and PCA analysis of both markers were able to distinguish the cultivars genetically. 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.

Key words: Diversity, ISSR, molecular markers, RAPD, dendgrogram, *Mangifera indica* L.

Key findings: Detection of genetic variation of 10 mango accessions collected from Vietnam by RAPD and ISSR molecular markers. The obtained results can be useful for plant identification and conservation of mango genetic resources.

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INTRODUCTION

Mango (*Mangifera indica* L.) is an important fruit tree, whose fruit contain high amount of amino acids, carbohydrates, fatty acids, minerals, organic acids, protein, ascorbic acid, carotenoids, phenolic compounds and vitamins. Mango belongs to Anacardiaceae family, consisting of at least 69 species and distributed in several countries in tropical region. Germplasm characterization and identification of genetic relatedness among accessions are important for conservation and breeding programs (Mansour *et al*., 2014). Traditional identification of mango mostly relies on morphological features such as locality, leaf-flower-flesh color, growth habit and other characteristics of plants. Although morphological classification is easy to perform and carry out on the field with low cost, there are several limitations for this method such as limited number, complex inheritance pattern and vulnerable to changes of environment (Ahmedand and Mohamed, 2014).

Molecular markers have been proven as a potential substitution with numerous advantages, such as unlimited in number, unaffected by environment and growing conditions, easy to interpret with reliable repeatable results (Antunes *et al*., 1997). Numerous molecular markers have been developed such as isozyme, Restriction Fragment Length Polymorphism (RFLP), Random Amplified of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple sequence Repeat (SSR), Inter-Simple sequence Repeat (ISSR), and Single Nucleotide Polymorphisms (SNP). Among which, RAPD and ISSR are highly preferred because they are PCR based markers and possess common advantages such as simple, rapid, economic, require minimum laboratory skill, require small DNA quantity, high number of fragments in each reaction, and do not require prior knowledge of genetic genome of targeted plants. RAPD has been extensively applied for several plants such as wheat (Nagaoka and Ogihara, 1997), cassava (Colombo *et al*., 2000), millet (Gupta *et al*., 2010), and bamboo (Desai *et al*., 2015). Study using RAPD for evaluating mango diversity were also carried out worldwide such as in China (Hou *et al*., 2005); Colombia (Díaz-Matallana *et al*., 2009); Brazil (Souza *et al*., 2011); Sudan (Ahmedand and Mohamed, 2014).

However, RAPD has its own limitations such as low reproducibility due to reactive conditions such as DNA concentrations, the concentration of PCR components, and the number of cycles of the reaction (Williams *et al*., 1990, Mbwana *et al*., 2006). ISSR is a preferred alternative as a highly variable, reproducible marker (Wolfe and Liston, 1998) leading to expanded use in genetic diversity research, population genetic studies, genetic markers, crop identification, source analysis, identification, genetic change identification, and cross-breeding in different plants such as potato (Bornet *et al*., 2002); sugar beet (Izzatullayeva *et al*., 2014); bitter gourd (Singh *et al*., 2015); millet (Dvořáková *et al*., 2015) and mango (Mansour *et al*., 2014; Rocha *et al*., 2012).

In Vietnam, mango has high market value and functions as a mean for poverty reduction in rural areas, make it as one of the most prioritized fruit plants for development. In 2016, General Statistics Office of Vietnam reported the growing areas were up to

74,600 ha with the production of 728,100 tons/year as (GSO, 2016). Also in this year, Vietnam Trade Office announced that Vietnam stood at 13th place of mango export countries (VTO, 2016). There is high number of cultivars and wild type varieties; however, there is a limited study to understand genetic composition of this plant. To date, however, only a few studies reported genetic composition of mango in this country. In 2012, Tran and Do analyzed genetic diversity of mango genotypes in Southern Vietnam by AFLP and IST markers (Tran and Do, 2012) and more recently, Le and colleagues used ISSR to study genetic variation of "thanhtra" (*Bouea oppositifolia* Roxb.), which is a member in mango family (Le *et al*., 2018). However, these studies only focused in Southern Vietnam. In this study, a total of 10 ISSR primers were used with objective to evaluate the genetic diversity of 10 mango samples from different regions of Vietnam. The obtained results could provide scientific information for identification, classification, propagation and breeding purposes.

MATERIALS AND METHODS

Plant materials

A total of ten mango seedlings with recorded identify were collected from germplasm of research institutes, university nurseries, and seedling centers of different provinces of Vietnam (Figure 1 and Table 1). After sampling, leaf 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 and Doyle (1990). DNA sample was stored at -20 °C until use. 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).

RAPD and ISSR amplification

A total of 10 RAPD (Yonemoto *et al*., 2006) and 10 ISSR primers (Levi *et al*., 2004) were used and shown in Table 2.

The composition of PCR reactions was performed as follows: 7.5 µL 2X Mytaq Red Mix (Bioline, UK), 20 ng DNA, 0.2 µM primer and PCR water for final volume of 15 µl. The RAPD 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 35°C, and 1 minute at 72°C. Finally, addition 5 minutes was continued at 72°C to complete the reaction. The ISSR reaction conditions were conducted as follows: initial denaturation at 95°C for 2 minutes; then 35 cycles of 30 seconds at 95°C, 30 seconds at 54°C, and 1 minute at 72°C and 5 minutes to finish reaction at 72°C. All reactions were carried out with the SureCycler 8800 Thermal Cycler (Agilent, USA).

Table 1. Mango samples collected for genetic characterization.

No.	Cultivar/variety/local name	Collected location	Sample code
1	XoaiUc	TienGiang	XU-TG
2	Xoaituquy	BinhThuan	XTQ-BT
3	Xoaitim	Ha Noi	XTI-HN
4	Xoai cat chu	Quang Nam	XCC-QN
5	Xoai cat	ThanhHoa	XC-TH
6	Xoaituong	Nghe An	XT-NA
7	Xoai rung	BinhPhuoc	XR-BP
8	Xoaituong	Ben Tre	XT-BT
9	Xoai cat HoaLoc	KhanhHoa	CHL-KH
10	Xoàithanhca	Dong Thap	XTC-ĐT

Table 2. List of RAPD and ISSR primers to analyze genetic diversity of 10 mango.

RAPD Primer	Sequence (5'-3')	ISSR Primer	Sequence (5'-3')
A39a	CCTGAGGTAGCT	UBC880	GGAGAGGAGAGGAGA
A58	GTCATGCCTGGA	UBC825	ACACACACACACACACT
Z1	CCGGTGCCTTCT	UBC841	GAGAGAGAGAGAGAGACTC
D41	GAGACCCGTCGA	UBC855	ACACACACACACACACCTT
D38	AAGCTCGACGGG	UBC813	CTCTCTCTCTCTCTCTT
D29	GACCCGGAACGA	UBC853	TCTCTCTCTCTCTCTCRT
A15	TTCCGAACCC	UBC809	AGAGAGAGAGAGAGAGG
C59a	CGCGTTCGTGGA	UBC814	CTCTCTCTCTCTCTCTA
A62	TCGTCCGGAGAT	UBC811	GAGAGAGAGAGAGAGAC
A18	GACTCGCGATCT	UBC810	GAGAGAGAGAGAGAGAT

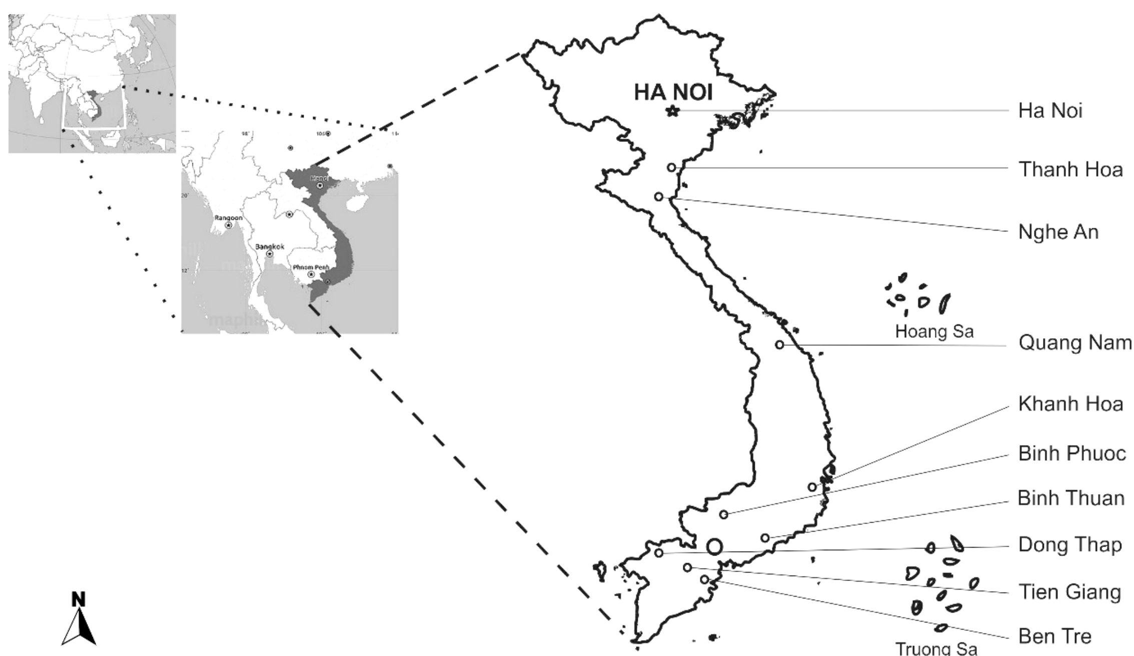

Figure 1. Targeted areas for collecting mango genotypes.

PCR amplification was then separated by electrophoresis in 1.5% agarose gel in 1X TAE buffer, and stained with 0.5 µg/ml Gelred TM loading buffer then visualized under UV transilluminator.

Data analysis

After gel electrophoresis, the clear amplification bands were used in the analysis. Only clear bands were analyzed; weak signal bands were excluded from final analysis. Since RAPD and ISSR are dominant markers, at each locus, the presence of amplified band was interpreted as either a heterozygote or dominant homozygote and the absence of a band in corresponding position as recessive homozygote (Debnath *et al*., 2008). Clearly visible ISSR amplified bands were scored as 1, whereas the absent band was scored as 0. The numbers of scored bands (SB), numbers of polymorphic bands (NPB) and percentage of polymorphic bands (PPB) were obtained. The quality information of the primers is determined by the PIC (Polymorphism Information Content) according to the formula of Chesnokov and Artemyeva (2015).

$PIC_j = 1-\sum_{i=1}^{n} Pi^2$, where i is the i th line of the primer j, n is the number of bands of the primer j, P is the frequency of the band. The ability of primers to differentiate between genotypes was evaluated by their resolving power (RP) value as describe by Prevost and Wilkinson (1999).

$RP = \sum_{i=1}^{n} BIi$, where n is the NPB of that primer and BI_i (In formativeness of a band) = 1-(2x|0.5-*p*|), p is the proportion of the 10 mango genotypes containing the band, IB value was calculated for 10 ISSR and 10 RAPD primers. Cluster analysis was performed by using Unweighted Pair Group Method with the Arithmetic mean (UPGMA). The SIMQUAL program was used to calculate the Jaccard's coefficients by using NTSYS-pc 2.1 (Rohlf, 2000). The Jaccard's coefficient was calculated as Rayar *et al* (2015). The dendrograms were constructed on the algorithm with the SAHN module in NTSYS-pc 2.1 (Rohlf, 2000). The cut-off values of the dendrograms were determined based on calculation method described by Jamshidi and Jamshidi (2011). Using similarity matrices, a Principal Coordinate Analysis (PCA) was carried out to construct a three-dimensional array of eigenvectors using DCENTER module of NTSYS-pc 2.1 program (Ibrahim *et al*., 2017). The Mantel test at a significant level of 5% in 1.0 permutation was conducted to identify the correlation between the RAPD and ISSR similarity matrices by using program Mantel test of Microsoft Excel 2010 (Mantel, 1967).

RESULTS

RAPD analysis

At first, the ten mango genotypes were characterized with 10 RAPD primers. The obtained results showed that the bands appeared clearly on 1.5% agarose gel (Figures 2A and Figure 2B). The tested primers generated from 11 to 23 amplifications per reaction and the length of amplifications varies approximately from 150 to 2,000 bps. The average number of bands and polymorphic bands per primer were

16.2 and 15.1, respectively. Percentage polymorphism ranged between 72.7% (primer Z1) to 100% (primer A39a; A15; and C59a), with an average of 91.9% across all analyzed mango genotypes. PIC value varied from 0.74 (primer A18) to 0.96 (primerA58) with the average of 0.89. The RP value varied from 7.6 (primer D41) to 14.4 (primer A62) (Table 3).

The matrix of Jaccard's coefficient showed similarity across genotypes (Table 4). The minimum similarity coefficient is 0.43, which is between CHL-KH and XT-NA, whereas the maximum similarity coefficient is 0.69, between XTQ-BT and XU-TG. The cluster analysis of genotypes is shown as a dendrogram in Figure 3A. A cut-off value of 0.57, the dendrogram consist of three major clusters. Cluster I comprised five genotypes XU-TG, XTQ-BT, XR-BP, XT-BT, and XT-NA. Cluster II consisted of three genotypes XTI-BT, XCC-QN, XC-TH. Cluster III included 2 genotypes CHL-KH and XTC-DT. The PCA analysis was performed to compare the cluster analysis and is shown in Figure 4A. The first three most informative components explained 42.15% of the total variation.

Based on the similarity matrix of 10 mango genotypes in Table 4, the phylogenetic tree was constructed and shown in Figure 3A.

ISSR analysis

Ten ISSR primers were used to evaluate genetic composition of 10 mango genotypes, the tested primers showed clear and reproducible bands (Figures 5A and Figure 5B). A total 145 bands were generated; out of which 143 bands were polymorphic, accounting for 98.1%. The sizes of amplified bands range from 200 to 2000 bps. The number of bands varied from 10 (primer UBC853) to 18 (primer UBC811). The average of amplified bands and polymorphic bands per primer were 14.5 and 14.3, respectively. Polymorphic percentage ranged between 92.3% (primer UBC825) to 100% (primer UBC880; UBC855; UBC813; UBC853; UBC809; UBC814; and UBC810), with an average of 98.1%. PIC value varied from 0.83 (primer UBC841) to 0.97 (primer UBC809) and RP value varied from 4.8 (primer UBC880) to 10.4 (primer UBC810) (Table 3).

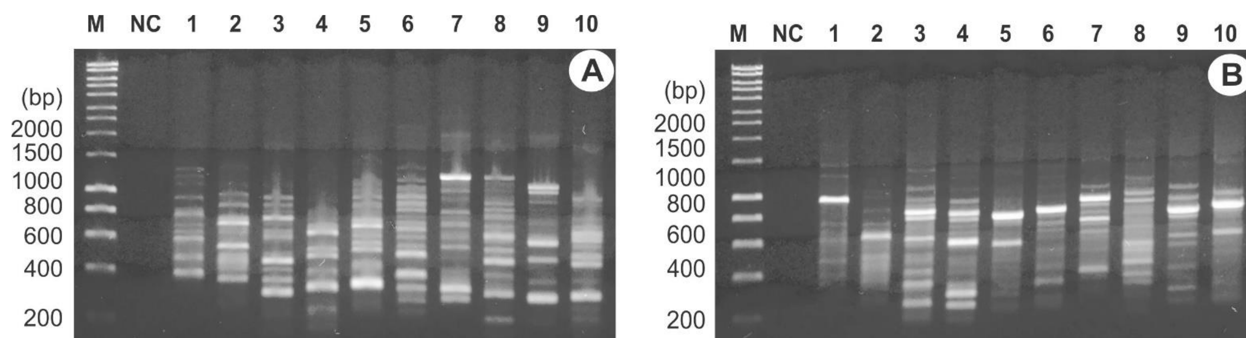

Figure 2: Representative RAPD results with C59a primer (A) and D38 primer (B). (The number is corresponding to sample number in Table 1; M: DNA marker; NC: Negative control without DNA).

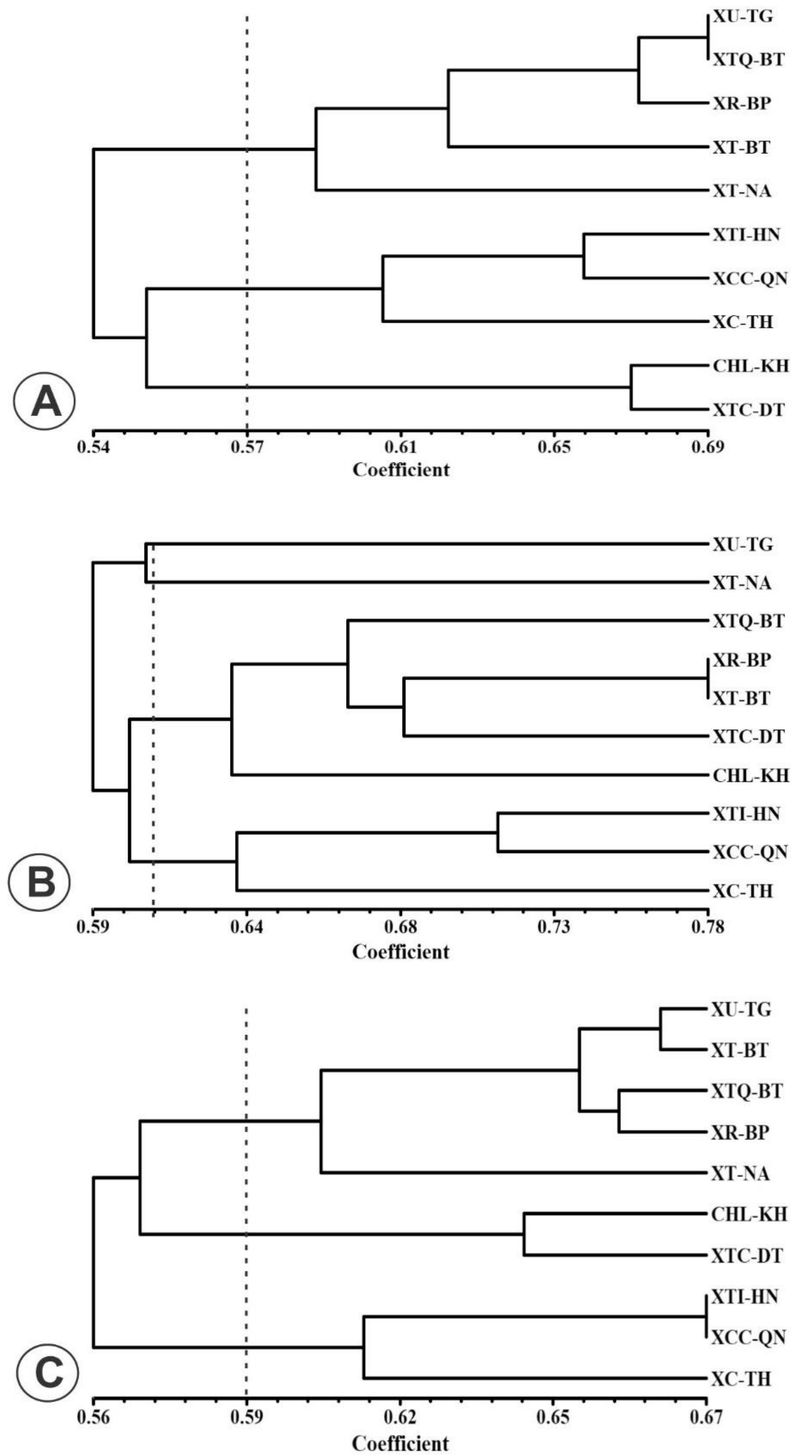

Figure 3: Dendrogram obtain from 10 genotypes of mango collected in Vietnam with UPGMA based on Jaccard's coefficient by using 10 RAPD primers (A); 10 ISSR primers (B) and 10 RAPD + 10 ISSR primers (C). The vertical lines indicate the cut-off values of each dendrogram and the scale shown at the bottom is the measure of genetic similarity through Jaccard's coefficient.

Based on UPGMA analysis with ISSR data, the dendrogram is constructed. At cut-off value of 0.61, the 10 genotypes were divided into four groups (Figure 3B). Groups I and groups II possess only one accession for each group namely XU-TG and XT-NA, respectively. Group III consists of five genotypes XTQ-BT, XR-BP, XT-BT, XTC-DT, and CHL-KH. Group IV includes three genotypes XTI-HN, XCC-QN, and XC-TH. The PCA analysis was performed to compare the cluster analysis and is shown in Figure 4B. The first three most informative components explained 42.15 of the total variation. According to Jaccard's coefficient matrix (Table 4), CHL-KH had the lowest coefficient with XT-NA (0.49) whereas XT-BT had the highest coefficient with XR-BP (0.79). The three most informative components explained up to 42.80% of the total variation.

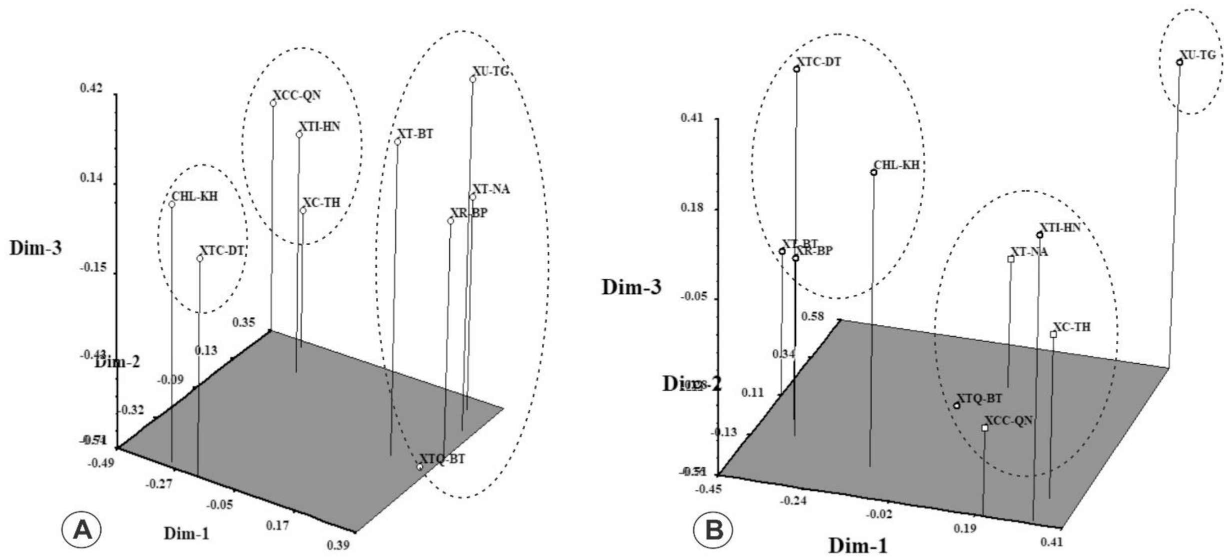

Figure 4: Three-dimensional plot of the principal coordinate (PCO) analysis of distance among 10 mango genotypes by using 10 RAPD primers (A) and 10 ISSR primers (B).

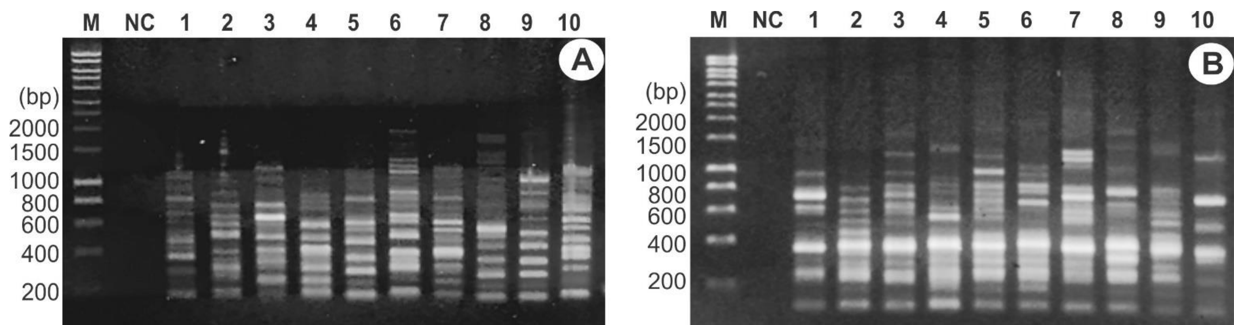

Figure 5: Representative ISSR result with UBC880 primer (A) and UBC811 primer (B). (The number is corresponding to sample number in Table 1; M: DNA marker; NC: Negative control without DNA).

Table 3. Characteristics of DNA profiles generated in 10 mango genotypes by using10 RAPD primers and 10 ISSR primers.

RAPD Primer	SB	NPB	PPB (%)	PIC	RP	ISSR Primer	SB	NPB	PPB (%)	PIC	RP
A39a	13	13	100	0.93	10.8	UBC880	16	16	100.0	0.91	4.8
A58	17	16	94.1	0.96	8,0	UBC825	13	12	92.3	0.85	6.8
Z1	11	8	72.7	0.93	12.4	UBC841	17	16	94.1	0.83	6.0
D41	12	10	83.3	0.91	7.6	UBC855	15	15	100.0	0.93	7.2
D38	16	14	87.5	0.92	7.8	UBC813	12	12	100.0	0.94	7.8
D29	13	12	92.3	0.78	8.4	UBC853	10	10	100.0	0.95	5.2
A15	18	18	100	0.95	10.2	UBC809	16	16	100.0	0.97	5.6
C59a	23	23	100	0.92	13.4	UBC814	14	14	100.0	0.88	5.2
A62	18	17	94.4	0.86	14.4	UBC811	18	17	94.4	0.86	8.6
A18	21	20	95.2	0.74	11.4	UBC810	14	14	100.0	0.93	10.4
Sum	162	151	-	-	-		145	143	-	-	-
Average	16.2	15.1	91.9	0.89	10.4		14.5	14.3	98.1	0.91	6.76

SB: Scored bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content; RP: resolving power.

Table 4: Simple matching coefficients of similarity among 10 mango genotypes with 10 RAPD primers (above diagonal) and ISSR (below diagonal).

	XU-TG	XTQ-BT	XTI-HN	XCC-QN	XC-TH	XT-NA	XR-BP	XT-BT	CHL-KH	XTC-ĐT
XU-TG		0.69	0.59	0.57	0.55	0.59	0.68	0.67	0.56	0.56
XTQ-BT	0.64		0.55	0.55	0.61	0.59	0.65	0.60	0.57	0.61
XTI-HN	0.61	0.58		0.66	0.58	0.51	0.55	0.53	0.56	0.57
XCC-QN	0.57	0.71	0.71		0.63	0.45	0.5	0.50	0.57	0.51
XC-TH	0.60	0.59	0.61	0.66		0.53	0.52	0.50	0.54	0.55
XT-NA	0.61	0.62	0.61	0.60	0.63		0.62	0.57	0.43	0.48
XR-BP	0.54	0.64	0.58	0.66	0.61	0.57		0.60	0.57	0.55
XT-BT	0.64	0.71	0.57	0.64	0.59	0.52	0.78		0.54	0.57
CHL-KH	0.57	0.62	0.61	0.61	0.55	0.49	0.59	0.69		0.67
XTC-ĐT	0.57	0.66	0.60	0.59	0.51	0.61	0.69	0.67	0.62	

Combined data

Then RAPD and ISSR data were combined and then subjected for UPGMA analysis. The Jaccard's coefficient is shown in Table 5. CBL-KH and XT-NA showed the lowest similarity (0.49) whereas XC-TH and XTI-HN present the highest similarity (0.67). The dendrogram based on the pooled data is shown in Figure 3C. At cut-off value of 0.59, the 10 genotypes were grouped into three majors. Cluster I comprise five genotypes XU-TG, XT-BT, XTQ-BT, XR-BP, and XT-NA. Cluster II consists of two genotypes CHL-KH and XTC-DT. Cluster III includes three genotypes XTI-HN, XCC-QN, and XC-TH. The cluster pattern of pooled data is more similar to RAPD cluster while the ISSR dendrogram showed some variation. Furthermore, as the similarity matrices of RAPD and ISSR markers were compared by using Mantel's test, the correlation between two matrices was low (0.27) and not significant.

Table 5. Simple matching coefficients of similarity among 10 mango genotypes with pooled data from RAPD and ISSR primers (below diagonal) and geographical distances in km (above diagonal).

	XU-TG	XTQ-BT	XTI-HN	XCC-QN	XC-TH	XT-NA	XR-BP	XT-BT	CHL-KH	XTC-ĐT
XU-TG		16.4	1,781	707	1,621	1,475	163	16.4	491	92.9
XTQ-BT	0.66		1,797	1,012	1,637	1,491	179	0	506	107
XTI-HN	0.59	0.56		797	160	298	1,515	1,797	1,291	1,576
XCC-QN	0.56	0.61	0.67		641	495	748	1,014	509	1,072
XC-TH	0.57	0.60	0.59	0.64		141	1,359	1,640	1,135	1,420
XT-NA	0.60	0.60	0.55	0.52	0.54		1,213	1,494	989	1,284
XR-BP	0.64	0.66	0.57	0.57	0.56	0.61		180	407	220
XT-BT	0.66	0.65	0.56	0.57	0.54	0.61	0.66		506	107
CHL-KH	0.56	0.59	0.59	0.59	0.55	0.49	0.57	0.60		565
XTC-ĐT	0.57	0.63	0.59	0.55	0.53	0.54	0.60	0.61	0.64	

DISCUSSION

RAPD and ISSR markers have been extensively used for cultivar identification and genetic characterization among mango accessions from different countries such as Brazil (Souza *et al*., 2011); Egypt (Mansour *et al*., 2014); India (Bajpai *et al*., 2016); Pakistan (Nazish *et al*., 2017). In this study, the potential ability of RAPD and ISSR markers was used to examine the genetic diversity of ten popular mango genotypes collected from different provinces of Vietnam. The results showed that RAPD makers generated more bands and polymorphic bands than those of ISSR marker. However, the percentage of polymorphic bands of ISSR is higher than RAPD. The complex marker patterns of ISSR will be advantageous for distinguishing closely related species (Rayar *et al*., 2015). Other studies also reported that ISSR primers are more informative than RAPD because of higher percentage of polymorphic band (Nagaoka and Ogihara, 1997; Rayar *et al*., 2015).

Both RAPD and ISSR primers showed high PIC values from 0.89 to 0.91 meaning that all these primers are suitable for studying genetic diversity in mango according to Botstein *et al*. (1980). Based on the obtained PIC value, it could suggest that an ISSR marker is more informative than RAPD. Moreover, ISSR is also considered more reproducible than RAPD markers by different studies (Goulão and Oliveira, 2001) since ISSR primers are longer and requires higher annealing temperature resulting better consistency. ISSR primers are also designed to specifically complement to corresponding microsatellite sequence leading less random annealing during PCR reaction.

The resolving power (RP) index was also to assess the ability to differentiate mango genotypes. The RP value varied from 4.57 for primer UBC855 to 8.57 for primer UBC813. In general, the primers showing higher RP value could distinguish more genotypes and show higher polymorphic bands (Debnath *et al*., 2008). This data also demonstrates that the primers used in the study could be amplified in highly genetically distinct regions between mango samples.

The obtained results from clustering analysis of RAPD data and

ISSR data were not similar. Their differences could be resulted from the working principle of each marker which anneals to different positions in plant genome. This data suggested that the variation was due to the characteristics of a specific genotype (Debnath *et al*., 2008). The PCA plotting presents similar grouping patterns to the cluster analysis for RAPD marker (Figure 3a and Figure 4a). Nevertheless, a large inconsistency was found for ISSR marker (Figure 3b and Figure 4b). Thus, the PCA analysis could reveal another level of separation of sample and it could be a benefit to use more than one type of analysis method. Although RAPD and ISSR are dominant markers, the Mantel test of the two matrices from RAPD and ISSR are of low correlation. The similar results have been reported with different plants such as potatoes (Rocha *et al*., 2012; Onamu *et al*., 2016), and wheat (Khaled *et al*., 2015). A reasonable explanation for this result could be the difference between the obtained data generated by two marker types when RAPD and ISSR amplify the non-repetitive and repetitive regions of plant genome (Ghislain *et al*., 2006).

The obtained data reveal that the studied mango accessions were not grouped as geographical location where samples were collected. Our result agrees with previous findings in different plants such as sweet potato (Gichuki *et al*., 2003) and potato (Moulin *et al*., 2012). This could be due to the exchanging of mango accession between neighboring farmers and relatives, resulting to the same genotype having different names in different location. In addition, the introduction of mango varieties from neighboring countries such as Taiwan, Malaysia also contributes to the genetic difference of the mango variety increase. Furthermore, the high diversity of collected samples in Vietnam could be due to other reasons such as out-crossing system or the geographical difference. Studies in chili (Albrecht *et al*., 2012) or olive (Mousavi *et al*., 2014) also reported that the high genetic diversity could be a cause of the adaptation of plants to specific geographical areas. The high genetic diversity of mango accessions collected in Southern Vietnam implies the diverse gene pool in this region. This genetic richness will be useful for mango breeding programs as the breeders could have more materials for expanding the genetic base of new mango cultivars which is necessary to enhance the yield and decrease the vulnerability of newly bred mango from several diseases and pests by combining superior traits from different genotypes. Furthermore, the distinguish DNA banding of each genotype could be used in molecular assisted-selection in breeding. This strategy could reduce significantly the number of required generations for traits/gene introgression and facilitate the rapid identification of genetically different parents to generate heterosis (Lavi *et al*., 2004).

In conclusion, both RAPD and ISSR are potential markers using for evaluating the genetic variation of mango genotypes. ISSR is more superior to RAPD because of the percentage of polymorphic bands and the high reproducibility. In the future, the development of Sequence Characterized Amplified Region (SCAR) marker should be considered to provide markers with higher level of authenticity for mango identification by using specific primers designed

from RAPD and ISSR potential amplicons.

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