ORIGINAL ARTICLE

Characterization of *Mangifera indica* cultivars in Thailand based on macroscopic, microscopic, and genetic characters

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ABSTRACT

Thai mango cultivars are classified into six groups plus one miscellaneous group according to germplasm database for mango. Characterization is important for conservation and the development of Thai mango cultivars. This study investigated macroscopic, microscopic leaf characteristics, and genetic relationship among 17 cultivars selected from six groups of mango in Thailand. Selected mango samples were obtained from three different locations in Thailand (n = 57). They were observed for their leaf and fruit macroscopic characteristics. Leaf measurement for the stomatal number, veinlet termination number, and palisade ratio was evaluated under a microscope attached with digital camera. DNA fingerprint was performed using CTAB extraction of DNA and inter-simple sequence repeat (ISSR) amplification. Forty-five primers were screened; then, seven primers that amplified the reproducible band patterns were selected to amplified and generate dendrogram by Unweighted Pair-Group Method with Arithmetic Average. These selected 17 Thai mango cultivars had individually macroscopic characteristics based on fruits and leaves. For microscopic characteristics, the stomatal number, veinlet termination number, and palisade ratio were slightly differentiable. For genetic identification, 78 bands of 190–2660 bps were amplified, of which 82.05% were polymorphic. The genetic relationship among these cultivars was demonstrated and categorized into two main clusters. It was shown that ISSR markers could be useful for Thai mango cultivar identification.

Key words: DNA fingerprint, inter-simple sequence repeat amplification, macroscopic character, *Mangifera indica* cultivars, microscopic leaf constants

INTRODUCTION

Mango (*Mangifera indica* L.), "Ayurveda king of fruits," is one of the most ancient and important tropical fruit in the world. It has been cultivated since at least 4000 years ago. It belongs to the *Anacardiaceae* family consisting of over 1000 cultivars.[1] In Thailand, mango has been cultivated since the early history of the Kingdom as many as 174 cultivars have been recorded.[2] Macroscopic- and microscopic-characterization should be the first step to identify the plants; they are primary important that should be carried out before any tests will be undertaken. Although they may be affected by the environmental conditions,[3,4] they are beneficial for botanical authentication.[5] In addition, DNA markers which less

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affected by age, physiological, and environmental conditions have been extensively developed as a powerful tool for plant identification and genetic relationship not only among species but also cultivars.[6,7] Simple sequence repeat (SSR), amplified fragment length polymorphism analysis, and a sequence analysis of the internal transcribed spacer have been reported for the potentials of cultivar identification of *M. indica* in Thailand.[8-11] Inter-simple sequence repeat (ISSR) primers were also valuable due to no genetic information need, rapidity, reproducibility, simplicity, and cost-effectiveness. ISSR could be performed to identify cultivars in many species.[12] This study aimed to investigate selected 17 Thai mango cultivars popularly cultivated in Thailand, on macroscopic and microscopic leaf characteristics as well as their genetic relationships using ISSR markers.

MATERIALS AND METHODS

Plant materials

Leaf samples of 17 Thai *M. indica* cultivars, *Mangifera Caloneura,* and *Bouea macrophylla* were collected during June to July in 2014. Each sample was collected from three locations per cultivar and authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Macroscopic characteristics

The observations using naked eyes on fruits (fruit shape) and leaves (leaf shape, leaf apex, leaf base, and leaf margin) were recorded.

Microscopic characteristics

A microscope (Axio Imager A2: Zeiss Inc., Germany) was used to observe stomatal number, veinlet termination, and palisade ratio under the objective lens magnification of ×20, ×5, and ×40, respectively and the eyepiece lens magnification of ×10. The microscope was attached to a Digital Camera (Power Shot A640: Cannon Inc., Japan) interfaced with a personal computer using an AxioVision40 software (V4.6.3.0: Zeiss Inc., Germany) for image labeling.

All mature leaf samples were cleaned and cut into small pieces approximately 10 mm × 5 mm in size. Calcium oxalates were removed, and tissues were disintegrated by poaching in 10% hydrochloric acid under low heat for 1 h. They were bleached with Haiter® solution (6% w/w Sodium Hypochlorite: Kao Corp., Japan), washed with water then cleared with 4 g/ml of chloral hydrate under low heat.

Leaf sample was mounted with a few drops of water. The selected cells were traced. Each sample was counted for 30 fields. The average of 90 fields from three locations per cultivar was carried out.

Molecular characteristics

Genomic DNA was extracted from the fresh young leaf tissues following a CTAB method[13] then determined for its purity by spectrophotometry and 1% agarose gel stained with 2 mg/ml of ethidium bromide, respectively.[14] Fragment size was estimated using GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc., USA).

ISSR amplification was performed according to Bornet and Branchard[15] with a minor modification; 45 primers (Eurofins MWG Operon Inc., USA) were screened, and primers that gave reproducible products were selected for further analysis. Polymerase chain reaction (PCR) amplifications were performed in 20 µl reaction mixtures; containing a final concentration about 50 ng of DNA, 2.5 mM of $MgCl_2$, 1X of PCR buffer, 0.1 µM of primer, 0.1 µM of each dNTP, and 0.5 unit of Taq DNA polymerase. ISSR amplifications were performed using a ProFlex PCR System Thermocycler (Thermo Fisher Scientific Inc., USA) with an initial denaturation step for 5 min at 95°C, followed by 45 cycles of denaturation step 45 s at 95°C, annealing step 45 s at annealing temperature of each primer, extension step 1 min at 72°C, and completed with a final extension for 5 min at 72°C. Optimal conditions were resolved based on ISSR-PCR products. A negative control, which contained all PCR mixture except genomic DNA, was included in every testing to evaluate the mixture contamination. ISSR amplified products were visualized on 1% agarose gel stained with 2 mg/ml of ethidium bromide.[14] Fragment size was estimated using GeneRuler 1 kb DNA ladder.

The reproducible amplified bands were chosen for analysis. Agarose gels were photographed (InGenius 3 with GeneSis Software, Syngene, UK), and fragment sizes were estimated (GeneTools Software, Syngene, UK). Amplification profiles were scored in binary code as present (1) or absent (0). A similarity matrix was analyzed, and a pairwise distance matrix was also generated a dendrogram by cluster analysis using Unweighted Pair-Group Method with Arithmetic Average (GeneDirectory Software, Syngene, UK) based on character differences.

RESULTS

Macroscopic characteristics

Observations on mango fruit and leaf macroscopic characteristics were reported listed in Table 1.

Microscopic characteristics

Mango stomata were anomocytic type which bordered by a varying number of cells and not different from the epidermis. They were small size and presented only in the lower surface of the leaf. The epidermal cells were oval or round shaped [Figure 1]. Stomata numbers were ranging from 515.11 to 954.58 stomata/1 mm^2, with an average of 695.82 stomata/1 mm^2. Mango leaf veins were netted veins patterns [Figure 1]; veinlet termination number was ranging from 24.69 to 45.08 veinlet terminations/1 mm^2, with an average of 36.41 veinlet terminations/1 mm^2. Mango palisade cells

lay between upper and lower epidermis [Figure 1]. Palisade ratio was ranging from 2.92 to 3.72, with an average of 3.23. The abundant fibers covering on *B. macrophylla* leaf caused their veinlet termination could not be detected [Table 2].

Molecular characteristics

Annealing temperatures, fragment sizes, total bands, polymorphic fragment, and polymorphic percentage of selected primers were summarized in Table 3.

Table 1: Macroscopic characteristics of selected *Mangifera indica* cultivars and outgroups

Group	Samples	Fruit shape	Leaf shape			
			Leaf shape	Leaf apex	Leaf base	Leaf margin
Nangklangwan	*M. indica* 'Nga Khao'	Cylindrical	Elliptical	Acute	Acute	Entire
Nangklangwan	*M. indica* 'Nangklangwan'	Cylindrical	Elliptical	Acute	Acute	Undulate
Namdokmai	*M. indica* 'Khiaoyai'	Elliptical	Lanceolate	Acute	Obtuse	Undulate
Namdokmai	*M. indica* 'Mankhunsi'	Cylindrical	Oblong	Acute	Acute	Undulate
Namdokmai	*M. indica* 'Namdokmai'	Elliptical	Oblong	Acuminate	Obtuse	Undulate
Nangklangwan	*M. indica* 'Mahacharnok'	Oblong	Linear-oblong	Acuminate	Obtuse	Undulate
Okrong	*M. indica* 'Kaemdaeng'	Elliptical	Lanceolate	Acute	Obtuse	Undulate
Okrong	*M. indica* 'Okrong'	Elliptical	Oblong	Acuminate	Acute	Undulate
Okrong	*M. indica* 'Chok Anan'	Obovate	Elliptical	Attenuate	Acute	Undulate
Okrong	*M. indica* 'Raet'	Obovate	Oblong-lanceolate	Attenuate	Obtuse	Undulate
Roundish	*M. indica* 'Talapnak'	Roundish	Oblong	Acute	Acute	Undulate
Kaeo	*M. indica* 'Kaeo'	Obovate	Elliptical	Acuminate	Acute	Entire
Khiaosawoey	*M. indica* 'Tongdam'	Obovate	Elliptical	Acute	Acute	Undulate
Khiaosawoey	*M. indica* 'Khiaosawoey'	Oblong	Oblong	Attenuate-acuminate	Attenuate	Undulate
Khiaosawoey	*M. indica* 'Falan'	Oblong	Linear-oblong	Acute	Acute	Entire
Kaeo	*M. indica* 'Phetbanlat'	Obovate	Oblong-lanceolate	Acuminate	Acute	Entire
Kaeo	*M. indica* 'Nongsaeng'	Oblong	Oblong-lanceolate	Acuminate	Acute	Entire
-	*M. caloneura*	Roundish	Oblong	Acute	Acute	Undulate
-	*B. macrophylla*	Ovoid	Ovate-oblong	Acute-acuminate	Acute-cuneate	Entire

Table 2: Leaf constant values of selected *Mangifera indica* cultivars and outgroups

Leaf samples	Stomatal number*,** (stomata/1 mm^2)	Veinlet termination number*,** (veinlet termination/1 mm^2)	Palisade ratio*
M. indica 'Nga Khao'	722.58±43.50	32.92±5.98	2.94±0.34
M. indica 'Nangklangwan'	594.76±166.21	36.51±3.80	3.36±0.32
M. indica 'Khiaoyai'	668.80±57.80	24.69±4.67	3.38±0.38
M. indica 'Mankhunsi'	622.22±42.47	32.47±4.35	3.72±0.42
M. indica 'Namdokmai'	515.11±33.37	29.35±3.45	2.98±0.44
M. indica 'Mahacharnok'	595.29±36.69	24.92±5.27	3.10±0.37
M. indica 'Kaemdaeng'	902.27±65.71	43.34±8.00	3.14±0.34
M. indica 'Okrong'	659.16±161.94	37.63±4.99	3.13±0.43
M. indica 'Chok Anan'	710.36±50.43	45.08±4.67	3.07±0.28
M. indica 'Raet'	954.58±52.41	43.13±4.36	2.92±0.30
M. indica 'Talapnak'	549.87±91.03	39.27±4.62	3.66±0.52
M. indica 'Kaeo'	803.38±125.90	44.56±10.24	3.14±0.35
M. indica 'Tongdam'	601.60±57.44	41.23±9.08	3.38±0.35
M. indica 'Khiaosawoey'	643.07±36.47	33.79±3.25	3.25±0.42
M. indica 'Falan'	844.09±53.67	33.80±3.30	3.55±0.46
M. indica 'Phetbanlat'	670.44±48.31	36.76±3.81	3.13±0.35
M. indica 'Nongsaeng'	771.42±56.21	39.48±5.26	3.11±0.38
M. caloneura	562.09±35.00	40.80±2.92	2.81±0.27
B. macrophylla	550.53±31.86	ND	1.94±0.21

*Mean±SD, **$P<0.05$ (ANOVA) was considered statistically significant differences among *Mangifera indica* cultivars. Data were the average of 90 determinations from three different locations per sample. ND: Could not detect, SD: Standard deviation

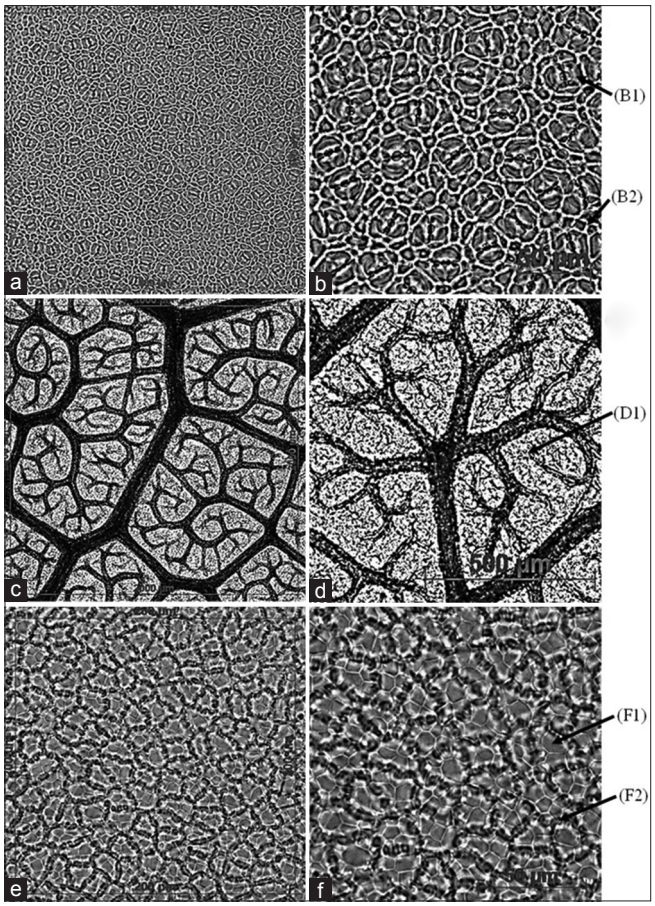

Figure 1: Images of *Mangifera indica* leaves showing (a) mango stomata at a magnification of × 200, scale 500 × 500 µm; (b) (B1) stomata cell and (B2) epidermal cell, scale 50 µm; (c) veinlet terminations at a magnification of × 50, scale 2000 × 2000 µm; (d) (D1) veinlet termination, scale 500 µm; (e) palisade and epidermal cells at a magnification of × 400, scale 200 × 200 µm; (f) (F1) stomata cell and (F2) epidermal cell, scale 50 µm

No band was found in negative control amplification. No ISSR primer amplified a unique band pattern among *M. indica* cultivars. The pattern that obtained from ISSR 31 was presented as an example in Figure 2.

The genetic similarity coefficients were calculated using Jaccard's coefficient.[16] Among *M. indica* cultivars, the highest genetic similarity value of 0.6985 was found between *M. indica* "Nga Khao" and "Nang klang wan;" whereas the lowest genetic similarity value of 0.0858 was found between *M. indica* "Mahacharnok" and "Talapnak" [Table 4].

The similarity coefficients generated the dendrogram, which separated different *M. indica* cultivars then grouped them into two major clusters. For the cluster I, the highest genetic similarity value of 0.6985 was found between *M. indica* "Nga Khao" and "Nang klang wan;" whereas, the lowest genetic similarity value of 0.1576 was found between *M. indica* "Namdokmai" and "Raet." For the cluster II, the highest genetic similarity value of 0.6397 was found between *M. indica* "Khiaosawoey" and "Falan;" whereas, the lowest genetic similarity value of 0.2544 was found between *M. indica* "Kaeo" and "Talapnak." *M. caloneura* and *B. macrophylla,* which were outgroups in this current study, were clearly separated from *M. indica* cultivars listed as the cluster III and IV, respectively [Figure 3].

DISCUSSION

M. indica is one of the prominent fruit crops of tropical and subtropical areas in the world. Its cultivars have confronted with confusions about numerous synonym nomenclatures and needed to be correctly identified.[17,18] Thai mango cultivars have been classified according to the fruit and leaf macroscopic characteristics.[19] In this study, 17 *M. indica*

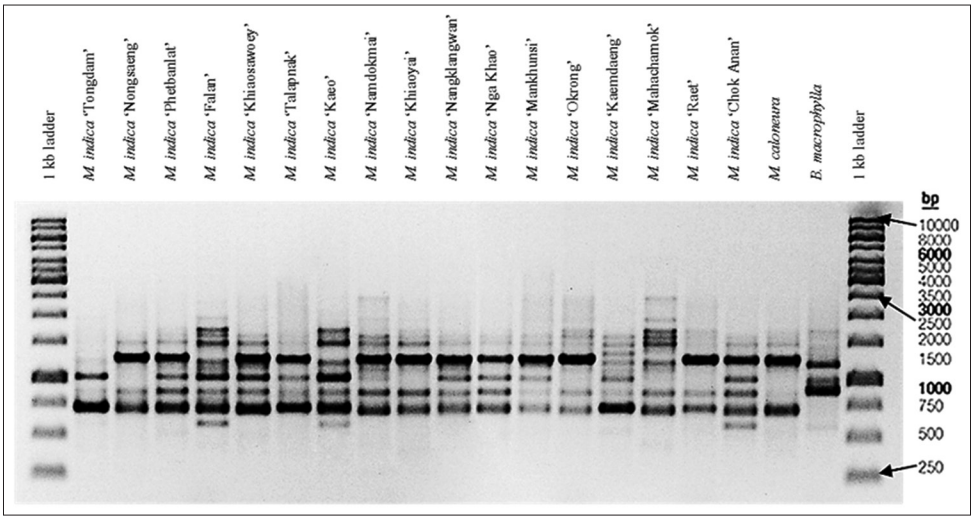

Figure 2: Inter-simple sequence repeat fingerprint of selected *Mangifera indica* cultivars and outgroups obtained from primer Inter-simple sequence repeat 31

Table 3: Summary of inter-simple sequence repeat markers

Primer	Primer sequence	Annealing Tm (°C)	Fragment size range (bps)	Total bands	Polymorphic fragment	Polymorphic percentage
ISSR02	AGAGAGAGAGAGAGAGC	50	380-2360	12	9	75.00
ISSR03	GAGAGAGAGAGAGAGAT	46	640-2560	13	12	92.30
ISSR13	AGAGAGAGAGAGAGAGYA	50	480-1760	9	7	77.78
ISSR19	ACACACACACACACACYT	54	650-1910	8	7	87.50
ISSR22	TGTGTGTGTGTGTGTGRC	54	360-2070	13	11	84.62
ISSR27	GGATGGATGGATGGAT	48	190-2660	11	9	81.82
ISSR31	AGAGAGAGAGAGAGT	44	570-2520	12	9	75.00
Total			190-2660	78	64	82.05

*Single letter abbreviations for mixed-base positions: Y=(C,T), R=(A,G)

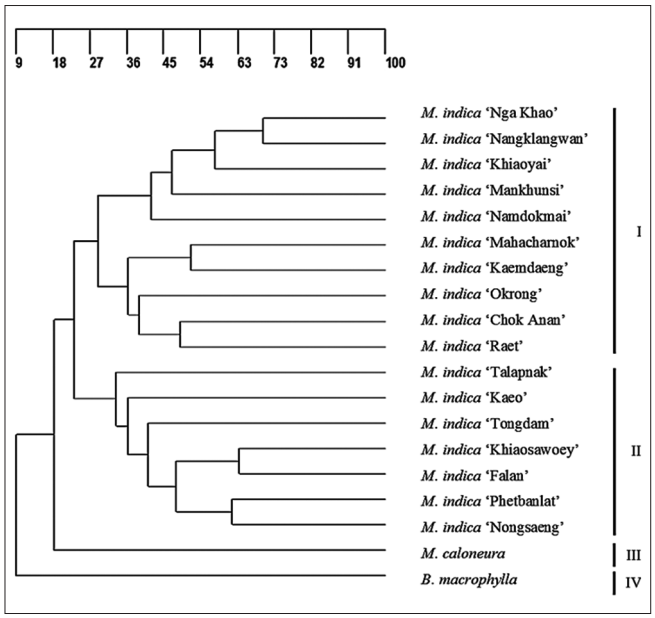

Figure 3: Dendrogram of *Mangifera indica* cultivars and outgroups using Unweighted Pair-Group Method with Arithmetic Average cluster analysis based on genetic similarities from selected seven Inter-simple sequence repeat primer

cultivars were selected from each group; Nang klang wan group ("Nga Khao," "Nang klang wan," "Mahacharnok"), Namdokmai group ("Khiaoyai," "Mankhunsi," "Namdokmai"), Okrong group ("Kaemdaeng," "Okrong," "Chok Anan," "Raet"), Roundish group ("Talapnak"), Keao group ("Kaeo," "Phetbanlat," "Nongsaeng"), and Khiaosawoey group ("Tongdam," "Khiaosawoey," "Falan"). These six groups have clear macroscopic characteristics. Nang klang wan group is cylindrical fruit shape with oblong leaf shape, attenuate leaf apex, and entire leaf margin. Namdokmai group is elliptical fruit shape with elliptical leaf shape, acuminate leaf apex, acute leaf base, and undulate leaf margin. Okrong group is elliptical fruit shape with lanceolate leaf shape, acuminate leaf apex, acute leaf base, and entire leaf margin. Roundish group is roundish fruit shape with elliptical leaf shape, attenuate leaf apex, acute leaf base, and entire leaf margin. Keao group is obovate fruit shape with lanceolate leaf shape, attenuate leaf apex, acute leaf base, and entire leaf margin. Khiaosawoey group is oblong fruit shape with oblong leaf shape, attenuate leaf apex, attenuate leaf base, and entire leaf margin.

Macroscopic characteristic assessments in fruit crops including mango typically require the presence of fruit. However, in the off-fruiting season, they still need to differentiate among those cultivars. Leaf microscopic and molecular characteristics can be used despite in off-fruiting season.[17] The stomatal patterning is variable among species, but it is regulated by a mechanism that sustains a minimum of one cell spacing between stomata.[20] Mango stomata were deep placed below the epidermis and presented only in the lower surface of a leaf (abaxial). They were anomocytic type. Their frequency was high, and their sizes were small, which allowed for immediate responses to prevent water loss.[21] Mango palisade cells, lie between the upper and lower epidermis, contain plentiful chloroplasts which response to light intensity. Palisade ratio was not varied based on geographical variation.[5] Mango leaf is netted veins pattern; major veins branch from the main ribs and subdivide into finer veinlets.

Leaf constant numbers could be used as distinguished characteristics of the plant. The results indicated that there were statistically significant differences in stomatal number and veinlet termination number among *M. indica* cultivars. Microscopic characteristic, as a supporting evidence, in combination with macroscopic and molecular characteristics was able to use as a helpful tool for more accurate identification.

Mango is a tropial diploid fruit crop ($2n = 40$ chromosomes), its genome size is about 4.39×10^8 base pairs.[22] Many AG, GA, AC, and CA dinucleotide repeat sequences were possible to exist in the mango genome because that repeat primer produced larger number of bands and polymorphic fragments.[23] GA and GT dinucleotide repeat sequences were also plenty present in the mango genome which could be effective to evaluate mango genetic diversity.[24] GACT and GGAT tetranucleotide repeat sequences were also found in the mango genome. In this study, most of the AG, GA, and TG dinucleotide repeat sequences and GGAT tetranucleotide repeat sequences were also successful in amplifying bands. The average polymorphic percentage (82.05%) in this study was higher than the other ISSR markers among mango

Table 4: Similarity index of *Mangifera indica* cultivars and outgroups

	M. indica 'Nga Khao'	*M. indica* 'Nangklangwan'	*M. indica* 'Khiaoyai'	*M. indica* 'Mankhunsi'	*M. indica* 'Namdokmai'	*M. indica* 'Mahacharnok'	*M. indica* 'Kaemdaeng'	*M. indica* 'Okrong'	*M. indica* 'Chok Anan'	*M. indica* 'Raet'	*M. indica* 'Talapnak'	*M. indica* 'Kaeo'	*M. indica* 'Tongdam'	*M. indica* 'Khiaosawoey'	*M. indica* 'Falan'	*M. indica* 'Phetbanlat'	*M. indica* 'Nongsaeng'	*M. caloneura*	*B. macrophylla*
M. indica 'Nga Khao'	1.0000																		
M. indica 'Nangklangwan'	0.6985	1.0000																	
M. indica 'Khiaoyai'	0.5119	0.6475	1.0000																
M. indica 'Mankhunsi'	0.5468	0.4889	0.3811	1.0000															
M. indica 'Namdokmai'	0.4222	0.3703	0.4812	0.4066	1.0000														
M. indica 'Mahacharnok'	0.3054	0.1867	0.3676	0.4293	0.2822	1.0000													
M. indica 'Kaemdaeng'	0.2733	0.2519	0.3718	0.3253	0.2516	0.5188	1.0000												
M. indica 'Okrong'	0.3128	0.2328	0.2893	0.3979	0.3117	0.3941	0.3439	1.0000											
M. indica 'Chok Anan'	0.3620	0.1937	0.2532	0.3036	0.2104	0.3525	0.3667	0.3683	1.0000										
M. indica 'Raet'	0.2942	0.2056	0.3054	0.3274	0.1576	0.3680	0.3563	0.4106	0.4921	1.0000									
M. indica 'Talapnak'	0.1335	0.1448	0.1406	0.1447	0.2577	0.0858	0.1284	0.2311	0.1852	0.1133	1.0000								
M. indica 'Kaeo'	0.3352	0.3619	0.3629	0.3377	0.4138	0.3177	0.3889	0.3066	0.2105	0.2475	0.2544	1.0000							
M. indica 'Tongdam'	0.2274	0.2226	0.2644	0.1792	0.3029	0.2785	0.2665	0.1871	0.2061	0.1542	0.3382	0.3555	1.0000						
M. indica 'Khiaosawoey'	0.2121	0.1613	0.2241	0.1997	0.2282	0.2517	0.2475	0.3533	0.2517	0.2161	0.4051	0.3585	0.3229	1.0000					
M. indica 'Falan'	0.1227	0.1141	0.1867	0.1622	0.2983	0.1905	0.1745	0.3513	0.2078	0.1717	0.3269	0.4483	0.3877	0.6397	1.0000				
M. indica 'Phetbanlat'	0.3026	0.2360	0.2225	0.2567	0.3222	0.2134	0.2527	0.3012	0.2294	0.1419	0.3946	0.3807	0.4725	0.5125	0.5712	1.0000			
M. indica 'Nongsaeng'	0.2703	0.2453	0.2602	0.2242	0.2015	0.2274	0.2039	0.1985	0.1421	0.1367	0.2843	0.2696	0.4654	0.4427	0.4078	0.6213	1.0000		
M. caloneura	0.1821	0.1539	0.1781	0.1900	0.1103	0.2401	0.2440	0.1787	0.3791	0.2673	0.1512	0.1171	0.1510	0.1376	0.0824	0.1722	0.1361	1.0000	
B. macrophylla	0.0925	0.0253	0.0384	0.0974	0.0510	0.0788	0.0798	0.0702	0.1235	0.1074	0.1235	0.1283	0.1319	0.1430	0.0853	0.0416	0.0702	0.0623	1.0000

cultivars in India (71.06%) and China (56.79%).[23,25] Although ISSR marker provided highly polymorphic percentage among these selected Thai mango cultivars, the number of total fragments amplified was relatively low. This might be because of electrophoretic gel types or staining technique influencing both number of total amplified band and polymorphic percentage detected. Polyacrylamide gel with silver staining may give more resolution.[12] No ISSR primer amplified a unique band pattern among *M. indica* cultivars. RAPD primer was alike; it was not amplified a unique band pattern also.[26]

CONCLUSION

This research revealed the macroscopic, microscopic, and molecular characteristics of selected 17 Thai mango cultivars. Macroscopic characters together with the dendrogram were sufficient to support dendrogram. ISSR had a potential to identify among 17 Thai mango cultivars. The dendrogram showed two major clusters. The cluster I was composed of 10 *M. indica* cultivars from three macroscopic characteristic groups; Nang klang wan group ("Nga Khao," "Nang klang wan," "Mahacharnok"); Namdokmai group ("Khiaoyai," "Mankhunsi," "Namdokmai"); Okrong group, ("Kaemdaeng," "Okrong," "Chok Anan," "Raet"). The highest genetic similarity of 0.6985 in the cluster I was found between "Nga Khao" (Nang klang wan group) and "Nang klang wan" (Nang klang wan group); whereas, the lowest genetic similarity of 0.1576 was found between "Namdokmai" (Namdokmai group) and "Raet" (Okrong group). The cluster II consisted of 7 cultivars from 3 macroscopic characteristic groups, Roundish group ("Talapnak"), Keao group ("Kaeo," "Phetbanlat," "Nongsaeng"), and Khiaosawoey group ("Tongdam," "Khiaosawoey," "Falan"). The highest genetic similarity of 0.6397 in the cluster II was found between "Khiaosawoey" (Khiaosawoey group) and "Falan" (Khiaosawoey group); whereas, the lowest genetic similarity of 0.2544 was found between "Kaeo" (Kaeo group) and "Talapnak" (Roundish group).

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Conflicts of interest

There are no conflicts of interest.

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