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...
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 Bormet 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 MgCl₂, 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 mm², with an average of 695.82 stomata/1 mm². Mango leaf veins were netted veins patterns [Figure 1]; veinlet termination number was ranging from 24.69 to 45.08 veinlet terminations/1 mm², with an average of 36.41 veinlet terminations/1 mm². 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 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      | Entire      |
| Namdokmai  | *M. indica* ‘Khiaoyai’ | Cylindrical | 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’| Oblong     | Elliptical | Attenuate  | Acute      | Undulate    |
| Okrong     | *M. indica* ‘Raet’     | Oblong     | Oblong-lanceolate | Attenuate-accumulate | Obtuse     | Undulate    |
| Roundish   | *M. indica* ‘Talapnak’ | Roundish   | Oblong     | Acute      | Acute      | Undulate    |
| Kaoe       | *M. indica* ‘Kaoe’    | Oblong     | Elliptical | Acuminate  | Acute      | Entire      |
| Khaosaowy  | *M. indica* ‘Tongdam’  | Oblong     | Elliptical | Acute      | Acute      | Undulate    |
| Khaosaowy  | *M. indica* ‘Khaosaowy’| Oblong     | Oblong     | Attenuate-accumulate | Attenuate | Undulate    |
| Khaosaowy  | *M. indica* ‘Falan’    | Oblong     | Linear-oblong | Acuminate  | Acute      | Entire      |
| Kaoe       | *M. indica* ‘Phetbanlat’ | Oblong     | Oblong-lanceolate | Acuminate  | Acute      | Entire      |
| Kaoe       | *M. indica* ‘Nongsaeng’| Oblong     | Oblong-lanceolate | Acuminate  | Acute      | Entire      |
| -          | *M. caloneura*         | Roundish   | Oblong     | Acute      | Acute      | Undulate    |
| -          | *B. macrophylla*       | Ovoid      | Ovate-oblong | Acuminate-accumulate | Acute-cuneate | Entire      |

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

| Leaf samples       | Stomatal number*,** (stomata/1 mm²) | Veinlet termination number*,** (veinlet termination/1 mm²) | 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* ‘Khaosaowy’ | 710.36±50.43 | 45.08±4.67 | 3.07±0.28 |
| *M. indica* ‘Falan’ | 954.58±52.41 | 43.13±4.36 | 2.92±0.30 |
| *M. indica* ‘Phetbanlat’ | 549.87±91.03 | 39.27±4.62 | 3.66±0.52 |
| *M. indica* ‘Nongsaeng’ | 503.36±125.90 | 44.56±10.24 | 3.14±0.35 |
| *M. indica* ‘Talapnak’ | 601.60±57.44 | 41.23±9.08 | 3.38±0.35 |
| *M. indica* ‘Kaoe’ | 543.07±36.47 | 33.79±3.25 | 3.25±0.42 |
| *M. indica* ‘Phetbanlat’ | 844.09±53.67 | 33.80±3.30 | 3.55±0.46 |
| *M. indica* ‘Nongsaeng’ | 771.42±56.21 | 36.76±3.81 | 3.13±0.35 |
| *M. indica* ‘Kaemdaeng’ | 670.44±48.31 | 39.48±5.26 | 3.11±0.38 |
| *M. indica* ‘Falan’ | 562.09±35.00 | 40.80±2.92 | 2.81±0.27 |
| *M. indica* ‘Phetbanlat’ | 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*
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* “Namokmai” 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* cultivars...
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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 entire 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. The stomatal patterning is variable among species, but it is regulated by a mechanism that sustains a minimum of one cell spacing between stomata. 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. Mango palisade cells, lie between the upper and lower epidermis, contain plentiful chloroplasts which respond to light intensity. Palisade ratio was not varied based on geographical variation. 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 tropical diploid fruit crop (2n = 40 chromosomes), its genome size is about 4.39 × 10^8 base pairs. 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. GA and GT dinucleotide repeat sequences were also plenty present in the mango genome which could be effective to evaluate mango genetic diversity. 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

| Primer  | Primer sequence            | Annealing Tm (°C) | Fragment size range (bps) | Total bands | Polymorphic fragment | Polymorphic percentage |
|---------|---------------------------|-------------------|---------------------------|-------------|----------------------|------------------------|
| ISSR02  | AGAGAGAGAGAGAGAC          | 50                | 380-2360                  | 12          | 9                    | 75.00                  |
| ISSR03  | GAGAGAGAGAGAGAT           | 46                | 640-2560                  | 13          | 12                   | 92.30                  |
| ISSR13  | AGAGAGAGAGAGAGYA          | 50                | 480-1760                  | 9           | 7                    | 77.78                  |
| ISSR19  | ACACACACACACACACYT        | 54                | 650-1910                  | 8           | 7                    | 87.50                  |
| ISSR22  | TGTGTGTGTGTGTGRC          | 54                | 360-2070                  | 13          | 11                   | 84.62                  |
| ISSR27  | GATGGATGATGGAT            | 48                | 190-2660                  | 11          | 9                    | 81.82                  |
| ISSR31  | AGAGAGAGAGAGAT            | 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)
### 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' | B. macrophylla |
|---------------------|--------------------------|----------------------|-----------------------|-----------------------|------------------------|----------------------|-------------------|---------------------|----------------|-------------------|----------------|------------------|---------------------|----------------|-------------------|------------------|----------------|
| 1.0000              |                         | 0.6985               | 0.5119                | 0.5468                | 0.4222                 | 0.3054               | 0.2733            | 0.3128              | 0.2942          | 0.1335            | 0.3526          | 0.2274            | 0.2121              | 0.1227          | 0.3026            | 0.2703            | 0.1821          |
|                     |                         | 1.0000               | 0.6475                | 0.3889                | 0.3703                 | 0.3676               | 0.3253            | 0.2893              | 0.2328          | 0.1937            | 0.2532          | 0.2644            | 0.2226              | 0.1867          | 0.2056            | 0.2602            | 0.1781          |
|                     |                         | 1.0000               | 1.0000                | 0.3811                | 0.4812                 | 0.4293               | 0.2822            | 0.3036              | 0.2732          | 0.2532            | 0.3036          | 0.1792            | 0.1997              | 0.1867          | 0.3054            | 0.2242            | 0.1867          |
|                     |                         | 1.0000               | 1.0000                | 1.0000                | 0.4066                 | 0.2822               | 0.2822            | 0.3036              | 0.3036          | 0.3563            | 0.3563          | 0.1576            | 0.1947              | 0.1576          | 0.3064            | 0.1622            | 0.1576          |
|                     |                         | 1.0000               | 1.0000                | 1.0000                | 1.0000                 | 0.3680               | 0.3680            | 0.3680              | 0.3680          | 0.4106            | 0.4106          | 0.3680            | 0.3680              | 0.3680          | 0.3680            | 0.3680            | 0.4106          |
|                     |                         | 1.0000               | 1.0000                | 1.0000                | 1.0000                 | 1.0000               | 1.0000            | 1.0000              | 1.0000          | 1.0000            | 1.0000          | 1.0000            | 1.0000              | 1.0000          | 1.0000            | 1.0000            | 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 “Nadmokmai” (Nadmokmai group) and “Raet” (Okrong group). The cluster II consisted of 7 cultivars from 3 macroscopic characteristic groups, Roundish group (“Talapnak”), Keao group (“Kaeo,” “Phethanlat,” “Nongsageng”), 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).

**Acknowledgments**
The authors would like to thank College of Public Health Sciences, Chulalongkorn University for instrumental support.

**Financial support and sponsorship**
The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) No. GCUGR1125592015D and Mae Fah Luang University Scholarship No. MFU002/2555.

**Conflicts of interest**
There are no conflicts of interest.

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