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Research article

Genetic and morphological diversity analysis of lime and acidic *Citrus* spp. from two germplasm collections in Thailand

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Abstract

Lime and other acidic *Citrus* fruits are cultivated and bred for commercial uses as ingredients in several types of foods and beverages as flavorings. To improve new cultivars through a better understanding of the genetic relationships of this type of *Citrus*, germplasm collections (50 accessions) were investigated consisting of Mexican lime, citron, lemon, Tahiti lime, kaffir lime, calamansi and finger lime obtained from the Tropical Fruit Research and Development Center and Ta Auan Lime Orchard, Thailand. Sequence-related amplified polymorphism (SRAP) markers and morphological traits were identified and used to classify the lime species and other acidic *Citrus* spp. cultivated in Thailand. Eleven SRAP markers were used to evaluate polymorphism and 340 polymorphic amplicons were observed with an average polymorphism information content of 0.45. Based on the results from the SRAP markers and population structure analysis, the 50 accessions of acidic *Citrus* trees in Thailand were divided into four subpopulations and classified into eight clusters that clustered the same types of *Citrus* together. Principal component analysis of acidic *Citrus* morphological data indicated close relatedness in accordance with genotypic data. This study revealed that Mexican lime (*C. aurantifolia* (Christm.) Swingle), the primary commercial lime grown in Thailand, has narrow genetic diversity. To improve lime varieties in the future, these findings can aid breeders in selecting more genetically diverse acidic *Citrus* species to cross with Mexican lime.

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Introduction

Citrus spp. are readily cultivated throughout tropical and sub-tropical parts of the world. Lime is an important ingredient in foods and beverages; consequently, the selection and breeding of lime cultivars has occurred for decades. Two lime species, Mexican lime (*C. aurantifolia* (Christm.) Swingle) and Tahiti lime (*C. latifolia* (Yu. Tanaka) Tanaka), are the most widely cultivated for commercial purposes that include fresh consumption and as an ingredient used by the food industry in Thailand, while in other countries, the lemon *C. limon* (L.) Osbeck is preferred.

To improve the quality of lime, *Citrus* genetic resources in Thailand can be used for the selection of desired characteristics according to market demand such as a thin fruit rind, good aroma and few seeds (Inglese and Sortino, 2019). However, the available Thai germplasm must be investigated first for both morphological traits and genetic diversity, as was done in several studies on local *Citrus* germplasm in Bhutan and Indonesia (Penjor et al., 2014; Penjor et al., 2016), southern Iran (Sharafi et al., 2016) and the Chuuk Islands of Micronesia (Yamamoto et al., 2018).

Various molecular markers in germplasm evaluation have been reported. Sequence-related amplified polymorphism (SRAP) markers were developed in 2001 by Li and Quiros using a polymerase chain reaction (PCR) molecular marker technique similar to random amplified polymorphic DNA (RAPD) markers (Li and Quiros, 2001). There have been several studies using SRAP markers to classify and cluster the genetic relationships of many organisms, including *Stipa bungeana* (Yu et al., 2014), *Malus* Mill. (Xu et al., 2014), *Miscanthus sinensis* (Nie et al., 2014), *Vitis ficifolia* Bge. (Fan et al., 2015), *Eucommoa ulmoies* Oliver (Yu et al., 2015), Japanese pagoda tree (*Styphnolobium japonicum* (L.) Schott) (Sun et al., 2016), bermudagrass (*Cynodon dactylon*) (Zheng et al., 2017) and ornamental purslane (*Portulaca* L.) (Jia et al., 2017).

Several marker types, including simple sequence repeat (SSR), cleaved amplified polymorphic sequence-single nucleotide polymorphism and SRAP markers, have been investigated regarding their ability to distinguish genetic relationships of *Citrus* germplasm and *C. aurantium* accessions, and it was revealed that SRAP markers had the highest polymorphism information content (PIC) among the markers used, indicating that SRAP markers are sufficient to analyse the diversity of organisms (Amar et al., 2011; Polat et al., 2012). Compared to SSR, inter-simple sequence repeat (ISSR), peroxidase gene polymorphism (POGP), resistant gene analog (RGA) and RAPD, the SRAP technique offers abundant amounts of markers which would be a benefit for using the marker to link to a trait of interest (Gulsen et al., 2010).

Citrus is thought to have originated from a region spanning northeastern India, northern Myanmar and northwestern Yunnan, from where lime is thought to have been brought to Southeast Asia (Wu et al., 2018) and was likely domesticated in Thailand. Mexican lime (*C. aurantifolia* (Christm.) Swingle) is a hybrid of *C. micrantha* Wester and *C. medica* L. (Curk et al., 2016) and is the most important commercial lime in Thailand. It is valued for its aroma and thin rind, making it easy to squeeze for juice. The propagation of Mexican lime in Thailand usually occurs using stem cutting, air layering, or grafting, in which bud mutation may cause diversity. On occasion, trees may be grown from seed, which may account for some of the diversity of Mexican lime in Thailand where the tree has developed from a zygote. In the current study, SRAP markers were used to study two collections of acidic *Citrus* germplasm and identify the genetic diversity of lime and *Citrus* germplasm in Thailand. Some morphological characteristics were also evaluated to show the variation in *Citrus* germplasm and the correlation of characteristics and genetics was discussed. The information of characteristics and genetic of acidic *Citrus* accessions in these germplasms can help lime breeding programs in the selection of parental lines to improve new varieties.

Materials and Methods

Plant materials and morphological and fruit quality characteristics

Samples of 50 acidic *Citrus* accessions grown at the Tropical Fruit Research and Development Center at the Kasetsart University, Kamphaeng Saen campus in Nakhon Pathom, Thailand and Ta Auan Farm Lime Orchard, Lopburi, Thailand, were collected in August 2018 (Table 1). All samples were grown in plant pots (dimensions 80 cm diameter and 40 cm depth) except samples of No. 35 that were using a tillage system. The mature fruit characteristics of each accession were recorded, except for those accessions that did not bear any fruit. Leaf characteristics were recorded at harvest. Three leaves and 1–10 fruits from 1–3 trees were sampled per accession. The characteristics observed and recorded were: leaf lamina attachment, petiole wing shape, skin color, fruit surface and flesh color according to the descriptors for *Citrus* (International Plant Genetic Resources Institute, 1999).

Table 1 List of acidic *Citrus* germplasm in Thailand used in this study

Accession number	Accession name	Scientific name	Type	Place of collection
1	Pan Phichit 1 #1	*C. aurantifolia* (Christm.) Swingle	Lime	TFRDC
2	Pan Yai #1	*C. aurantifolia* (Christm.) Swingle	Lime	TFRDC
3	Pan Sirinon	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
4	Pan Phichit 1 #2	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
5	Pan Phichit 2	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
6	Pan Phichit 4	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
7	Pan Jamrus 28	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
8	Pan Jamrus 29	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
9	Pan Rampai	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
10	Pan Pohkhun Sukhothai	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
11	Pan Yai #2	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
12	Pan Maelookdok	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
13	Pan Chaopraya	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
14	Pan Jamras 9 #1	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
15	Pan Jamras 9 #2	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
16	Pan Malay	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
17	Pan Sookprasert	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
18	Pan Praewa	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
19	Pan Dokpiset	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
20	Pan Iamseng	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
21	Thornless Lime	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
22	Manow Prachin	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
23	Dokdubai	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
24	Lime Sunspine	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
25	Pan Dankwian	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
26	Maekai Khaidok	*C. aurantifolia* (Christm.) Swingle	Lime	Ta Auan Farm
27	Citron	*C. medica* L.	Citron	TFRDC
28	Buddha's Hand Citron	*C. medica* L.	Citron	Ta Auan Farm
29	Pumpkin Lime	*C. medica* L.	Citron	Ta Auan Farm
30	Lemon Hawaii	*C. limon* (L.) Osbeck	Lemon	Ta Auan Farm
31	Lemon Eureka	*C. limon* (L.) Osbeck	Lemon	Ta Auan Farm
32	Nampetch	*C. latifolia* (Yu.Tanaka) Tanaka	Lime	Ta Auan Farm
33	Tahiti	*C. latifolia* (Yu.Tanaka) Tanaka	Lime	Ta Auan Farm
34	Toonklao	*C. latifolia* (Yu.Tanaka) Tanaka	Lime	Ta Auan Farm
35	Toonklao Namhom	*C. latifolia* (Yu.Tanaka) Tanaka	Lime	Ta Auan Farm
36	Petch Phongam	*C. latifolia* (Yu.Tanaka) Tanaka	Lime	Ta Auan Farm
37	Kaffir Lime #1	*C. hystrix* DC.	Kaffir lime	TFRDC
38	Kaffir Lime #2	*C. hystrix* DC.	Kaffir lime	TFRDC
39	Seedless Kaffir Lime	*C. hystrix* DC.	Kaffir lime	Ta Auan Farm
40	Australian Golden Sweet Lemon	*Citrus* sp.	Orange	Ta Auan Farm
41	Sweet Lime	*Citrus* sp.	Orange	Ta Auan Farm
42	Somlorddood	*Citrus* sp.	Orange	Ta Auan Farm
43	Unknown	*Citrus* sp.		Ta Auan Farm
44	Manow Yak	*Citrus* sp.		Ta Auan Farm
45	Manow Phee	*Citrus* sp.	Lemon	Ta Auan Farm
46	Variegated Calamansi	*Citrus*×*microcarpa* Bunge	Calamansi	Ta Auan Farm
47	Finger Lime #1	*C. australasica* F.Muell.	Finger lime	TFRDC
48	Finger Lime #2	*C. australasica* F.Muell.	Finger lime	TFRDC
49	Finger Lime #3	*C. australasica* F.Muell.	Finger lime	TFRDC
50	Finger Lime #4	*C. australasica* F.Muell.	Finger lime	TFRDC

TFRDC = Tropical Fruit Research and Development Center, Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Thailand

Aroma, total soluble solids (TSS), titratable acid (TA), fruit diameter (D), fruit height (H), the ratio of fruit diameter to fruit height (D/H index) for the indication of fruit shape, seed number and seed embryony were also recorded. The aroma was recorded based on the initial smell after cutting a fresh fruit. TSS were measured using a digital handheld refractometer (Atago; Tokyo, Japan). TA was determined using 2 mL of lime juice with 0.3 mL of 1% phenolphthalein added as an indicator, which was titrated with 0.1N sodium hydroxide until the color changed to a pink that persisted for 30 s (Association of Official Analytical Chemists, 2000). Fruit diameter and height were measured using a set of digital Vernier calipers (Insize; Suzhou New District, China). The seed number per fruit was counted after the juice and seeds had been extracted from a fruit. Seed embryony was observed by sowing the seeds in peat moss and observing the seedlings after germination for about 2 months. Mature leaves of all accessions were collected for DNA extraction and marker analysis.

DNA extraction and sequence-related amplified polymorphism analysis

Fresh mature leaves of all accessions were used for DNA extraction. Total DNA was extracted using the CTAB method (Doyle and Doyle, 1987) and stored at -20°C until use. The SRAP analysis of acidic *Citrus* genomic DNA was carried out using the forward primers (ME1–ME5) and reverse primers (EM1–EM5) developed by Li and Quiros (2001).

The PCR reaction mixture of 20 µL consisted of 100 ng of DNA, 0.25 µM of each forward and reverse primer, 1x reaction buffer and 1 unit of ExcelTaq™ *Taq* DNA polymerase (SMOBIO; Hsinchu, Taiwan). PCR reactions were adapted from Li and Quiros (2001) to increase the stringency of primer binding and performed as follows: initial denaturation at 94°C for 3 min, 38 cycles of denaturation at 94°C for 1 min, annealing at 46°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PCR products were separated using gel electrophoresis on a 6% polyacrylamide gel (Bio Basic Canada Inc.; Markham, Ontario, Canada) and were stained with silver nitrate (EMSURE®; Boston, MA, USA). The resulting bands were scored as either present (1) or absent (0).

Data analysis

The examination of genetic relationships among accessions was performed using cluster analysis based on a genetic similarity matrix produced with an unweighted pair-group method with arithmetic mean (UPGMA) method using the PAST version 3 software (Hammer et al., 2013). Population structure was inferred using the STRUCTURE version 2.3.4 software (Pritchard et al., 2000). Analyses were performed with the K value varying between 1 and 10. The ΔK value was optimized for the K value of the population as explained by Evanno et al. (2005). STRUCTURE was run 10 times with 50,000 burn-in steps followed by 50,000 Monte Carlo Markov Chain repetitions. Principal components analysis (PCA) with quality and quantity data were scored and standardized using PAST version 3.

Results

Acidic citrus germplasms in Thailand and their morphological traits

From the 50 accessions of acidic *Citrus* germplasm, 9 accessions did not bear any fruit (Fig. 1 and Table 2); the morphological analysis on the remainder revealed that 26 accessions resembled Mexican lime (*C. aurantifolia* (Christm.) Swingle), 5 accessions were Tahiti lime (*C. latifolia* (Yu.Tanaka) Tanaka), 3 accessions were kaffir lime (*C. hystrix* DC.), 4 accessions were similar to an orange or orange hybrid (*Citrus* spp.), 3 accessions were similar to lemon (*C. limon* (L.) Osbeck), 3 accessions corresponded to citron (*C. medica* L.) and 4 accessions were finger lime *(C. australasica* F.Muell.). Two accessions, 'Manow Yak' and 'Unknown', could not be identified due to the difficulty of categorization based solely on their morphology because 'Manow Yak' had large fruit like a pummelo but the TA content was higher than pummelo (Kongsri and Nartvaranant, 2019), whereas 'Unknown' did not bear any fruit (Table 1).

The 26 accessions resembling Mexican lime, except for accessions 10 and 25, all had a smooth fruit surface. Most had obloid fruit shapes because the fruit diameter was greater than the fruit height (D/H ≥ 1) except for 'Thornless Lime', 'Pan Rampai' and 'Maekai Khaidok' that had spheroid fruit with the fruit diameter almost equal to the fruit height (D/H=1) and 'Lime Sunspine' had an ellipsoid fruit shape with the fruit diameter less than the fruit height (D/H ≤ 1). These four accessions also tended to have fewer seeds than

the other obloid-shaped accessions of Mexican lime (Table 2). The leaves of all 26 accessions were a brevipetiolate type (Table 2).

Principle component analysis was used to study the leaves and fruits for 41 of the 50 accessions since 9 of the accessions did not bear any fruit (accession numbers 10, 25, 27, 39, 43, 47, 48, 49 and 50). From the results, the first two PCs accounted for 48.31% of total variability (PC1 = 29.12% and PC2 = 19.19%, Fig. 2). On PC1, the fruit skin color, fruit height and fruit surface had high loading scores of 0.85, 0.72 and 0.67, respectively (Table 3). In addition, seed number and fruit diameter impacted on PC2 separation with loading scores of 0.71 and 0.64, respectively (Table 3). The 24 accessions of Mexican lime were clustered together, whereas the accessions with a larger fruit size (accessions 28, 29, 40, 42 and 44) and yellow- or orange-colored peel (accessions 30, 31, 41 and 46) were in a distinct cluster. The accession numbers that resembled Tahiti limes (accessions 32, 33, 34, 35 and 36) also formed a distinct cluster.

Fig. 1 Morphological characters of acidic *Citrus* fruit genetic resources in Thailand included in the present analysis, where scale bar =2 cm

Table 2 Leaf and fruit characteristics (Mean ± SD) of acidic *Citrus* germplasm in Thailand

No.	Accession	Leaf lamina attachment*	Petiole wing shape†	Skin color‡	Fruit surface§	Flesh color‖	Aroma	Total soluble solid (°Brix)	Titratable acid (%)	Diameter (Hammer et al.)	Height (Hammer et al.)	D/H index¶	Seed number	Seed embryony#
1	Pan Phichit 1 #1	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.9±0.4	6.2±0.2	49.7±4.3	46.6±4.3	1.1±0.1	26.4±7.2	Poly
2	Pan Yai #1	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.6±1.1	4.6±1.2	46.7±3.9	41.2±3.9	1.1±0.0	29.0±9.6	Poly
3	Pan Sirinon	Brevipetiolate	Obdeltate	Green-yellow	Smooth	Green	Lime	8.0±0.8	4.7±1.8	43.1±3.8	37.1±3.3	1.2±0.0	11.0±1.4	-
4	Pan Phichit 1 #2	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.3±0.6	5.5±1.2	45.7±4.9	42.9±5.4	1.1±0.1	27.3±11.6	Poly
5	Pan Phichit 2	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.6±1.3	6.1±1.5	47.9±2.9	44.0±2.8	1.1±0.0	23.4±2.0	Poly
6	Pan Phichit 4	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.5±0.4	3.9±0.2	45.1±0.8	41.5±0.9	1.1±0.0	24.8±1.7	Poly
7	Pan Jamrus 28	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.4±0.2	6.2±0.2	43.8±3.0	39.8±1.8	1.1±0.0	23.4±4.5	Poly
8	Pan Jamrus 29	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.4±1.1	7.0±0.3	48.3±3.1	42.7±2.8	1.1±0.0	25.6±6.3	Poly
9	Pan Rampai	Brevipetiolate	Obdeltate	Green	Smooth	Green	Lime	7.7±0.7	6.0±0.7	36.9±2.0	37.4±2.6	1.0±0.0	7.9±8.1	Poly
10	Pan Pohkhun Sukhothai	Brevipetiolate	Obovate	-	-	-	Lime	-	-	-	-	-	-	-
11	Pan Yai #2	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.6±1.0	4.9±1.0	48.2±5.3	42.5±4.4	1.1±0.1	26.4±6.3	Poly
12	Pan Maelookdok	Brevipetiolate	Obovate	Green-yellow	Smooth	Green	Lime	7.7±0.2	7.1±0.3	41.9±2.0	36.1±1.1	1.2±0.0	11.1±3.1	Poly
13	Pan Chaopraya	Brevipetiolate	Obovate	Green	Smooth	Yellow	Lime	9.0±0.8	6.9±0.3	45.7±2.2	40.5±3.9	1.1±0.1	10.5±2.8	Poly
14	Pan Jamras 9 #1	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.3±0.6	7.1±0.5	51.0±3.2	44.8±2.8	1.1±0.0	34.0±6.1	Poly
15	Pan Jamras 9 #2	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.6±0.6	5.7±1.9	47.8±3.8	42.0±2.8	1.1±0.0	31.5±7.4	Poly
16	Pan Malay	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	6.9±1.7	6.6±0.2	40.1±3.8	35.9±3.1	1.1±0.0	13.3±3.1	Poly
17	Pan Sookprasert	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	10.2±1.2	8.5±1.0	38.7±2.1	36.8±2.5	1.1±0.0	13.6±2.3	Poly
18	Pan Praewa	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.5±0.5	6.9±1.2	44.7±2.4	40.2±6.1	1.1±0.2	29.9±5.4	Poly

Table 2 Continued

No.	Accession	Leaf lamina attachment*	Petiole wing shape†	Skin color‡	Fruit surface§	Flesh color‖	Aroma	Total soluble solid (°Brix)	Titratable acid (%)	Diameter (Hammer et al.)	Height (Hammer et al.)	D/H index¶	Seed number	Seed embryony#
19	Pan Dokpiset	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.1±0.6	4.9±1.5	46.0±1.3	39.8±0.9	1.2±0.0	24.3±5.8	Poly
20	Pan Iamseng	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	8.0±0.6	4.9±1.0	47.2±3.0	42.9±2.5	1.1±0.0	32.0±4.7	Poly
21	Thornless Lime	Brevipetiolate	Obovate	Green	Smooth	Yellow	Lime	7.5±0.4	3.4±0.2	38.3±1.4	37.3±2.1	1.0±0.0	11.0±0.0	-
22	Manow Prachin	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	-	-	28.0±0.0	23.5±0.0	1.2±0.0	-	-
23	Dokdubai	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	7.6±0.3	4.8±1.1	44.7±2.9	38.9±3.2	1.2±0.1	22.7±11.3	Poly
24	Lime Sunspine	Brevipetiolate	Obovate	Green	Smooth	Yellow	Lime	8.1±0.4	5.7±2.0	35.9±2.7	38.6±2.6	0.9±0.1	3.0±1.4	Poly
25	Pan Dankwian	Brevipetiolate	Obovate	-	-	-	-	-	-	-	-	-	-	-
26	Maekai Khaidok	Brevipetiolate	Obovate	Green	Smooth	Green	Lime	9.2±0.4	6.2±2.4	35.2±1.9	34.0±2.9	1.0±0.1	2.4±1.5	-
27	Citron	Sessile	-	-	-	-	-	-	-	-	-	-	-	-
28	Buddha's Hand Citron	Sessile	-	Yellow	Grooved	-	-	-	-	59.6±0.0	98.1±0.0	0.6±0.0	0.0±0.0	-
29	Pumpkin Lime	Sessile	-	Yellow	Grooved	White	Lime	6.8±1.3	4.6±1.5	89.0±24.2	67.7±22.5	1.3±0.1	71.5±26.2	Mono
30	Lemon Hawaii	Sessile	-	Yellow	Smooth	Yellow	Lime	8.0±1.2	4.7±0.9	49.9±3.5	76.9±9.4	0.7±0.0	7.4±2.1	Mono
31	Lemon Eureka	Sessile	-	Yellow	Smooth	Yellow	Lime	8.5±0.7	6.3±0.5	45.2±2.2	66.9±4.0	0.7±0.0	16.3±14.7	Mono
32	Nampetch	Brevipetiolate	Obdeltate	Green	Smooth	Green	Lime	8.4±0.4	6.1±2.7	49.5±5.4	52.4±6.6	0.9±0.0	0.0±0.0	-
33	Tahiti	Brevipetiolate	Obcordate	Green	Smooth	Green	Lime	8.9±0.6	6.5±1.1	44.6±1.8	48.1±3.3	0.9±0.0	0.0±0.0	-
34	Toonklao	Brevipetiolate	Obdeltate	Green	Smooth	Green	Lime	9.3±1.2	5.2±2.4	44.5±3.3	46.6±2.3	1.0±0.0	0.0±0.0	-
35	Toonklao Namhom	Brevipetiolate	Obdeltate	Light yellow	Smooth	Yellow	Lime	9.1±0.9	6.3±0.2	42.3±2.8	40.3±3.9	1.1±0.1	2.1±1.2	-
36	Petch Phongam	Brevipetiolate	Obdeltate	Green	Smooth	Green	Lime	10.5±1.4	5.9±1.3	46.4±3.5	49.2±3.9	0.9±0.0	0.0±0.0	-
37	Kaffir Lime #1	Longipetiolate	Obovate	Green	Papillate	Green	Kaffir lime	10.0±0.8	4.1±0.5	47.0±6.4	52.6±9.7	0.9±0.1	28.1±9.4	Mono
38	Kaffir Lime #2	Longipetiolate	Obovate	Green	Papillate	Green	Kaffir lime	9.2±0.3	7.9±0.6	51.3±9.5	59.7±13.1	0.9±0.1	20.8±6.1	Mono
39	Seedless Kaffir Lime	Longipetiolate	Obovate	-	-	-	-	-	-	-	-	-	-	-
40	Australian Golden Sweet Lemon	Sessile	-	Green	Smooth	Yellow	Orange	7.6±0.6	3.3±0.8	68.5±4.9	79.0±4.4	0.9±0.0	11.3±4.3	Mono
41	Sweet Lime	Sessile	-	Green, orange	Rough	Orange	Orange	8.6±0.6	0.3±0.2	51.6±4.3	49.3±5.5	1.1±0.1	9.4±3.1	-
42	Somlorddood	Brevipetiolate	Obcordate	Green	Rough	White	Orange	9.1±0.9	3.2±0.7	82.7±6.9	71.2±5.0	1.2±0.0	35.8± 9.5	Poly
43	Unknown	Sessile	-	-	-	-	-	-	-	-	-	-	-	-
44	Manow Yak	Brevipetiolate	Obovate	Yellow	Rough	White	Lime	7.4±0.6	4.3±1.3	97.9±9.1	100.2±11.6	1.0±0.1	3.8±3.6	Mono
45	Manow Phee	Sessile	-	Green-yellow	Smooth	White	Lime	8.0±0.6	6.1±0.8	53.8±2.8	55.8±4.7	1.0±0.1	13.0±10.5	Mono
46	Variegated Calamansi	Sessile	-	Green, orange	Smooth	Orange	Orange	8.2±0.5	4.1±0.9	31.4±1.4	30.1±1.2	1.0±0.0	3.8±1.8	Poly
47	Finger Lime #1	Sessile	-	-	-	-	-	-	-	-	-	-	-	-
48	Finger Lime #2	Sessile	-	-	-	-	-	-	-	-	-	-	-	-
49	Finger Lime #3	Sessile	-	-	-	-	-	-	-	-	-	-	-	-
50	Finger Lime #4	Sessile	-	-	-	-	-	-	-	-	-	-	-	-

Standards for each characteristic are:

* = leaf lamina attachment: sessile (petiole absent); brevipetiolate (petiole shorter than leaf lamina); longipetiolate (petiole longer than or same length as leaf lamina).

† = petiole wing shape: obcordate; obdeltate; obovate

‡ = skin color: green; green-yellow; light yellow; yellow; dark yellow; light orange; orange.

§ = fruit surface: smooth; rough; papillate; pitted; bumpy; grooved.

‖ = flesh color: white; green; yellow; orange.

¶ = fruit shape: D/H ≤ 1 is ellipsoid; D/H = 1 is spheroid; D/H ≥ 1 is obloid.

= seed embryony: mono; poly.

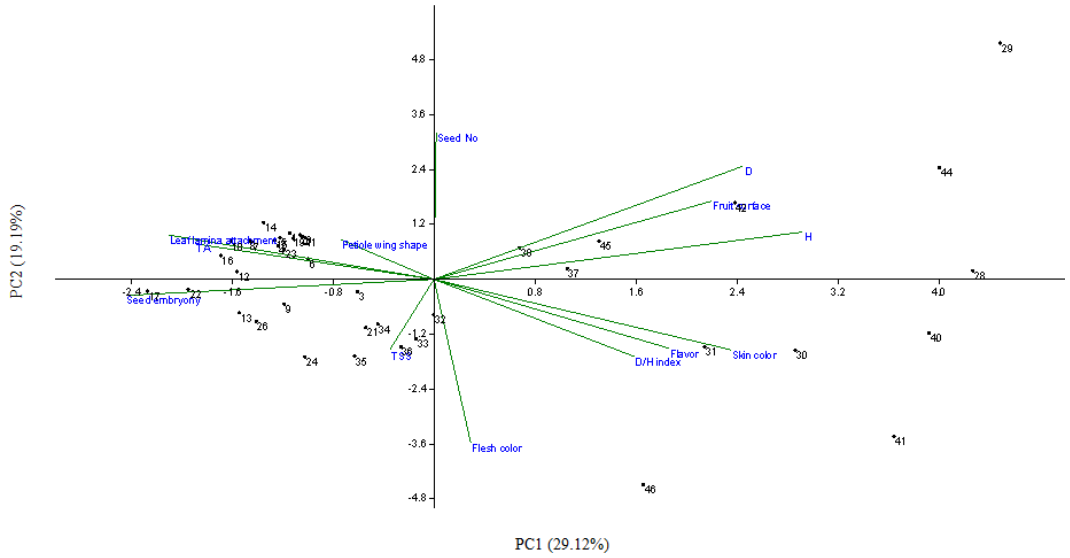

Fig. 2 Principal component (PC) analysis results based on leaf lamina attachment, petiole wing shape, skin color, fruit surface, flesh color, flavor, total soluble solid (TSS), titratable acid (TA), fruit diameter (D), fruit height (H), D/H index, seed number, and seed embryony for 41 accessions of *Citrus* spp.

Table 3 Loading scores of each principal component (PC) for PC 1 and PC 2

Characteristic	PC 1	PC 2
Leaf lamina attachment	-0.6405	0.239
Petiole wing shape	-0.1063	0.08035
Skin color	0.847	-0.433
Fruit surface	0.6652	0.4773
Flesh color	0.1811	-0.8087
Aroma	0.5014	-0.2181
TSS	-0.1677	-0.259
TA	-0.5641	0.1332
D	0.6238	0.642
H	0.7224	0.3621
D/H index	0.2751	-0.2167
Seed No	0.03449	0.7112
Seed embryony	-0.5436	-0.1524

TSS = total soluble solid; TA = titratable acid; D = diameter; H = height

Sequence-related amplified polymorphism analysis of acidic Citrus genetic resources in Thailand

From the 25 SRAP primer pairs, 11 primer combinations produced clear and diverse amplified bands, and polymorphisms were observed (Table 4). In total, 352 bands were displayed with an average of 32 bands per primer pair ranging from 16 (ME4EM4) to 52 (ME5EM5) bands. The number of total polymorphic fragments was 340 with a mean of 30.9. The PIC values ranged from 0.36 (ME1EM2) to 0.50 (ME2EM2, ME4EM4 and ME5EM4) with a mean of 0.45.

The population structure was analyzed using the STRUCTURE program for K values ranging from 1 to 10. Analysis of ΔK showed that the optimal results were obtained with K = 4 (Fig. 3A). Using the SRAP results for 50 accessions, the average gene diversity (H) was calculated. The H value of the population was 0.40, suggesting that there was genetic variation in this germplasm collection (Table 3). The H values of subpopulations ranged from 0.14 to 0.29. The highest diversity value was in subpopulation 2 and the lowest (0.14) was in subpopulation 1 (having only one type of *Citrus*).

Table 4 List of 11 combinations of sequence-related amplified polymorphism (SRAP) primer pairs used in this study

SRAP primer combination	Bands generated	Number of polymorphic bands	PPB (%)	PIC
ME1EM2	31	26	83.9	0.36
ME1EM3	40	38	95	0.44
ME2EM1	20	20	100	0.46
ME2EM2	26	26	100	0.5
ME3EM3	30	30	100	0.46
ME4EM2	31	31	100	0.42
ME4EM4	16	16	100	0.5
ME5EM1	37	37	100	0.44
ME5EM3	33	29	87.9	0.49
ME5EM4	36	35	97.2	0.5
ME5EM5	52	52	100	0.39
Total	352	340	96.6	-
Mean	32	30.9	96.7	0.45

PPB = percentage polymorphic band; PIC = polymorphism information content

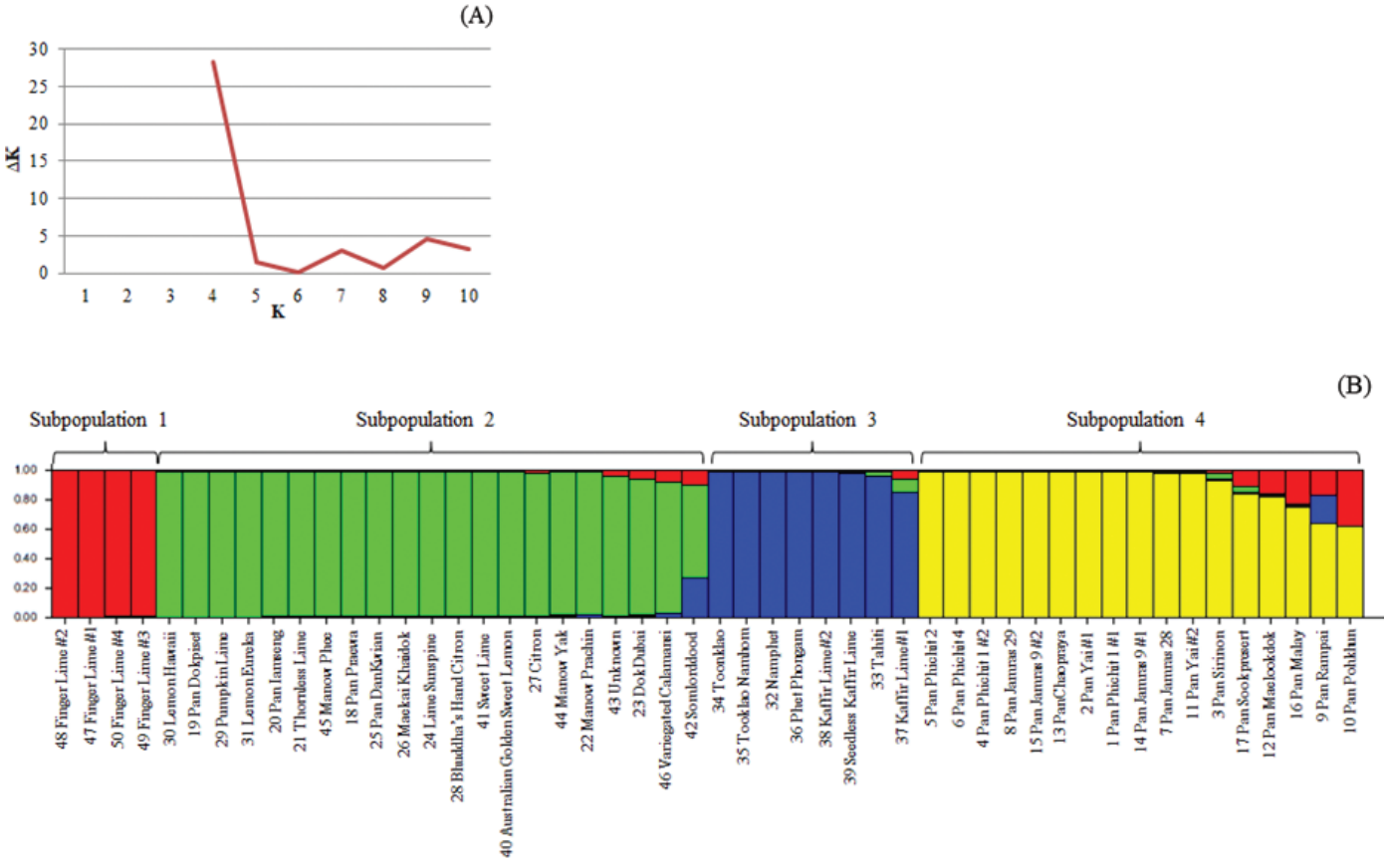

Fig. 3 Population structure of 50 acidic *Citrus* accessions based on sequence-related amplified polymorphism markers data for K= 4, where each color represents one subpopulation: (A) number of subpopulations indicated by highest ΔK value; (B) clustering of individuals into four subpopulations

A dendrogram was plotted using UPGMA clustering derived from the SRAP data on the results of the genotype calculated according to the Dice (1945) coefficient, as shown in Fig. 4. The 50 accessions were divided into eight clusters at a similarity coefficient of 0.60.

Discussion

The present study used morphological and molecular markers to characterize acidic *Citrus* germplasm grown in Thailand. The combined results of morphological and molecular markers offered valuable information for the identification of *Citrus* genetic resources and for *Citrus* breeding programs in Thailand.

SRAP markers are efficient for studying the diversity of germplasm. The present study used 11 combinations of primers to amplify in 50 accessions of acidic *Citrus* germplasm, obtaining 352 bands for each amplification with high average percentage polymorphic band (96.7%) and PIC (0.45) values (Table 4), consistent with the study on 24 accessions of *Citrus* germplasm using SRAP with 33 combinations of primers that produced 704 bands with 93% polymorphic bands (Amar et al., 2011). Another study on 51 accessions of sour orange germplasm using 21 primer combinations obtained

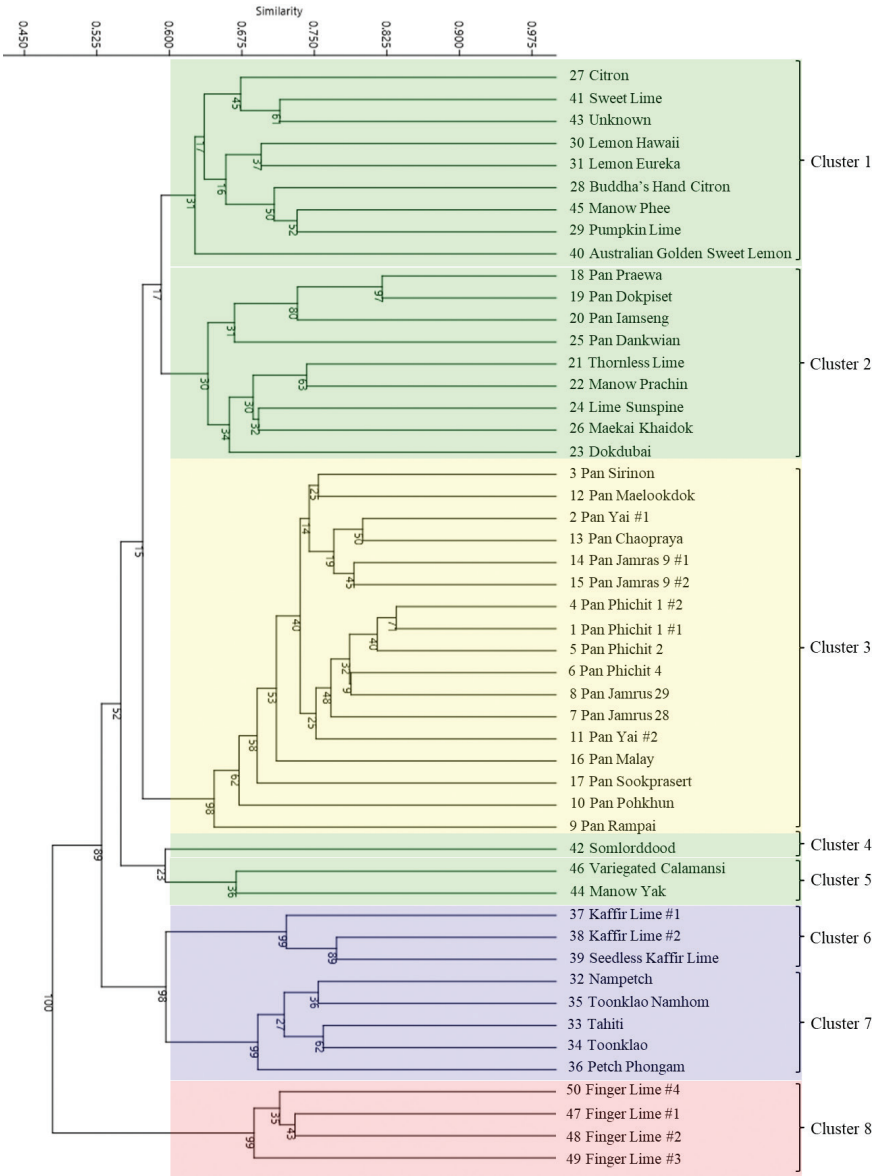

Fig. 4 Unweighted pair-group method of arithmetic averages dendrogram generated from sequence-related amplified polymorphism data showing relationships of 50 acidic *Citrus* accessions

87% polymorphic bands from 192 bands (Polat et al., 2012). The high amounts of markers obtained from the SRAP technique can increase the genome coverage that would be a benefit for linkage mapping (Gulsen et al., 2010).

The Mexican lime and Tahiti lime could be differentiated morphologically and were clearly distinguished in the DNA analysis (Figs. 1 and 4). The results of the UPGMA and population structure analyses showed that 17 accessions of Mexican lime were grouped together, specifically in cluster 3 on the UPGMA dendrogram and in subpopulation 4 in the STRUCTURE analysis. However, nine accessions fell into cluster 2 in the UPGMA and subpopulation 2 in STRUCTURE analysis, but the bootstrap values of the branches that separated clusters 1, 2 and 3 were low at 15 and 17 (Fig. 3 and 4). Mexican lime is a hybrid of *C. micrantha* Wester and *C. medica* L. (Curk et al., 2016; Wu et al., 2018) and when self- or cross-pollination between Mexican limes occur, variation may be created. The Mexican limes in cluster 2 were more mixed with both obloid, spheroid and ellipsoid shapes, while cluster 3 contained mainly obloid shapes which is a fruit type more preferred by consumers and hence breeding of lime in the past may have been based on the obloid shape. Cluster 3 (subpopulation 4) was composed of commercial Thai varieties such as accessions 1, 4, 5 and 6, whereas cluster 2 (subpopulation 2) consisted of non-commercial Mexican limes. The similarity coefficient in cluster 3 of 0.65–0.83 revealed that the genetic similarity of the Mexican limes in this germplasm collection was relatively high, perhaps due to a few common accessions of Mexican lime being selected to cultivate on a commercial scale, resulting in little genetic diversity (Fig. 4). PCA was used to generate an overview of the correlation among the samples based on leaf and fruit characteristics. The PCA results (Fig. 2) showed that the Mexican limes were grouped closely together with a similar morphology but they were quite separate from the other citrus species.

The Tahiti lime is also a seedless type or having non-germinating seeds since it is a triploid hybrid of *C. limon* (L.) Osbeck (1x ovule) and *C. aurantifolia* (Christm.) Swingle (2x pollen) (Curk et al., 2016). The variation observed in Tahiti lime and Mexican lime could also be caused by bud mutation since the genetics of Tahiti lime and Mexican lime were highly similar in their clusters (Fig. 4). However, the SRAP technique produced a lower estimate of genetic diversity because it cannot distinguish between diploid and triploid.

Nonetheless, citron, lemon and Mexican lime were on the same node showing relatively close relatedness likely due to the Mexican lime being the F1 hybrid of micrantha and citron, while lemon is the hybrid of sour orange and citron (Curk et al., 2016). Just as for variation in Mexican lime, variation in lemon can arise both through bud mutation or segregation of its zygote.

Relatives of orange were identified based on morphological traits, including aroma, peel and flesh color. 'Sweet lime' had the lowest TA among the 41 accessions with the fruit and its flesh having an orange color (Table 2). However, the genetic analysis clustered it with 'Citron', indicating that 'Sweet lime' is probably an interspecific hybrid of citron and the orange (Fig. 4). 'Australian Golden Sweet Lemon' was clustered with citron and lemon and is probably an interspecific hybrid of orange and lemon (Fig. 4). 'Somlorddood' was in a separate cluster from the others, but it was genetically close to 'Variegated Calamansi' and 'Manow Yak'. While 'Manow Yak' is morphologically similar to *C. maxima* (Burm.) Merr., except it was high in TA. Therefore, 'Somlorddood' and 'Manow Yak' may be relatives of *C. maxima* (Burm.) Merr. and some orange species (Fig. 4). 'Variegated Calamansi' is *Citrus x microcarpa* Bunge based on its characteristic. The 'Unknown', based on the phylogenetic analysis, was closely related to 'Citron' and 'Sweet lime'. Although the dendrogram separated 'Sweet lime', 'Unknown', 'Australian Golden Sweet Lemon', 'Somlorddood', 'Variegated Calamansi' and 'Manow Yak' into different clusters, the STRUCTURE analysis indicated that they were in the same subpopulation (Figs. 3 and 4).

The kaffir lime-types of 'Kaffir Lime #1', 'Kaffir Lime #2' and 'Seedless Kaffir Lime' were considered to be true *C. hystrix* DC. based on the results of the morphological and molecular marker data, which showed that they separated into a distinct cluster (Figs. 1 and 4). However, in the STRUCTURE analysis, kaffir limes were grouped with Tahiti lime in the same subpopulation (Fig. 3). The analysis of the genetic relationship of *C. hystrix* DC. Based on the RAPD and SCAR markers showed that it is closely related to *C. micrantha* Wester, a progenitor of Tahiti lime (Nicolosi et al., 2000). In the present study, the TSS contents of kaffir lime and Tahiti lime were closely related, which was fairly well supported by the genetic data and fruit quality of these two groups. The genetic analysis of the four finger-lime accessions revealed that they all were closely related (Figs. 3 and 4).

Morphological similarities among *Citrus* spp. are not always predictive of their genetic similarities and *vice versa*. Crossing other acidic limes or *Citrus* from different genetic backgrounds may help generate variety in lime cultivars in Thailand in the future to improve some fruit qualities of *Citrus*, such as seedlessness, improved juice aroma, peel thickness or flesh color. The present investigation of the current *Citrus* germplasm showed that the genetic base of Mexican lime used in Thailand is narrow. By providing a better understanding of the available genetic resources, the present findings may facilitate improved parent selection within *Citrus* breeding programs in Thailand.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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