Cultivar identification and genetic relationship of pineapple (*Ananas comosus*) cultivars using SSR markers

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ABSTRACT. The genetic relationships among 27 pineapple [*Ananas comosus* (L.) Merr.] cultivars and lines were examined using 16 simple sequence repeat (SSR) markers. The number of alleles per locus of the SSR markers ranged from 2 to 6 (average 3.19), for a total of 51 alleles. Similarity coefficients were calculated on the basis of 51 amplified bands. A dendrogram was created according to the 16 SSR markers by the unweighted pair-group method. The banding patterns obtained from the SSR primers allowed most of the cultivars and lines to be distinguished, with the exception of vegetative clones. According to the dendrogram, the 27 pineapple cultivars and lines were clustered into three main clusters and four individual clusters. As expected, the dendrogram showed that derived cultivars and lines are closely related to their parental cultivars; the genetic relationships between pineapple cultivars agree with the genealogy of

their breeding history. In addition, the analysis showed that there is no obvious correlation between SSR markers and morphological characters. In conclusion, SSR analysis is an efficient method for pineapple cultivar identification and can offer valuable informative characters to identify pineapple cultivars in Taiwan.

Key words: *Ananas comosus*; Cultivar identification; Dendrogram; Molecular marker; Pineapple; Simple sequence repeat marker

INTRODUCTION

Pineapple [*Ananas comosus* (L.) Merr.], belonging to the family Bromeliaceae, is a perennial herbaceous fruit tree that produces the third most important commercial fruit crop in the world (Botella and Smith, 2008). It is a diploid fruit tree with 2n = 2x = 50 chromosomes (Smith and Downs, 1979). The plant originated in Brazil, Paraguay, Central and South America. Pineapple cultivars are usually obtained through open pollination and seedling selection (Smith and Downs, 1979) or mutation breeding (Maluszynski et al., 1995).

Modern pineapples originated in pre-Columbian times in South America (Purseglove, 1972). Hume and Miller (1904) divided varieties in Florida into three types, Cayenne, Queen, and Red Spanish, on the basis of the general similarity of morphological characters. Py and Tisseau (1965) and Leal and Soule (1977) further separated Pernambuco and Maipure (Perolera) types from the others, for a total of five horticultural types of pineapple varieties. Although many pineapple cultivars have been bred and described, only a few have been commercialized. As these cultivars have been extensively transferred and have been acclimated in many different countries, they frequently have been renamed. Furthermore, geographical differentiation, clone selection, and cultivar heterogeneity contribute to the confusion. As a result, the classification of pineapple cultivars is very difficult. Many different cultivars are known by the same name and many different names may be given to the same cultivar (Leal, 1990).

Recently, several DNA profiling techniques have been used to identify cultivars and evaluate genetic diversity in pineapple cultivars. Restriction fragment length polymorphism (RFLP) markers have been used to detect chloroplast DNA polymorphism in pineapple (Duval et al., 2001), random amplified polymorphic DNA markers have been used to analyze 18 germplasms of pineapple (Ruas et al., 2001), and amplified fragment length polymorphism (AFLP) markers have been used to clarify the intraspecific DNA polymorphisms of pineapple (Kato et al., 2005). Simple sequence repeats (SSRs) are molecular markers based on tandem repeats of short (2-6 bp) DNA sequences (Litt and Luty, 1989). The copy number of repeats is highly polymorphic, even among closely related genotypes (Brown et al., 1996). The codominant and high polymorphic characteristics of microsatellite loci make them useful in cultivar identification (Chiou et al., 2012; Tsai et al., 2013) and hybrid evaluation (Liao et al., 2012; Chiang et al., 2013). Microsatellite markers (SSRs) of pineapple have been developed and used widely due to their high polymorphism and genome specificity (Kinsuat and Kumar, 2007). SSRs in expressed sequence tags (EST-SSRs) have been developed to analyze cross-amplification in pineapple at the species, genus, subfamily, and family levels (Wöhrmann and Weising, 2011). SSR markers have also been used to discriminate pineapple cultivars in Japan (Shoda et al., 2012).

Pineapple is one of the most important economic fruit crops in Taiwan and its cultivation covers around 9030 ha (Kuan et al., 2012). A breeding program for pineapple has been conducted

at the Kagi Agricultural Experiment Station in Taiwan since 1926 (Janick and Moore, 1996). During cultivation and propagation, several different types of cultivars and lines have been used as sources of specific characteristics. To establish effective breeding strategies, it is necessary to understand the genetic relationships among pineapple cultivars and to protect new cultivars of pineapple by using molecular markers. In the present study, 16 SSR primers were used to investigate the genetic relationships among 27 pineapple cultivars and lines in Taiwan.

MATERIAL AND METHODS

Plant materials

A total of 27 pineapple samples were collected and cultivated at the Department of Life Sciences, National Chung Hsing University, Taiwan (Table 1).

DNA extraction and PCR amplification

Genomic DNA was extracted from mature leaf powder using the protocol from the Plant Genomic DNA Miniprep System Kit (Viogene, Taipei, Taiwan). A total of 11 (Acom series) and 5 (EST-SSR series) polymorphic SSR markers were derived from Wöhrmann and Weising (2011) and Carlier et al. (2012), respectively, in order to evaluate the genetic relationships of the 27 pineapple cultivars and lines in Taiwan. The designed forward primers for the 16 SSR markers were elongated from the M13 (-21) 18-bp sequence (5'-TGTAAAACGACGGCCAGT-3') by fluorescent labeling (Schuelke, 2000). The designed primer pairs were first tested for PCR amplification and then used to amplify the 27 pineapple cultivars and lines after optimization. PCR conditions were as follows: total volume 25 µL with 20 ng of template DNA, 1X PCR buffer, 0.2 mM of each dNTP, 0.2 mM of each SSR specific primer, and 0.25 U *Taq* DNA polymerase (Promega, Madison, WI, USA).

Two-step PCR amplification was conducted. The first thermocycling profiles were initial denaturation at 94°C for 3 min, followed by 20 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C, 40 s extension at 72°C, and a final extension for 7 min at 72°C. After that, 0.075 mM M13 primer 5'-labeled with IRDye was added to the PCR mixture. The second thermo cycling profiles were initial denaturation at 94°C for 3 min, followed by 10 cycles of 30 s denaturation at 94°C, 30 s annealing at 58°C, 40 s extension at 72°C, and a final extension for 7 min at 72°C. Samples were denatured in loading dye (10 mg/mL blue dextran in formamide) and separated using 6.5% polyacrylamide gel (19:1,7 M urea) electrophoresis in a LI-COR 4300 DNA analyzer (LI-COR, Lincoln, NE, USA). Fragment lengths were determined with the aid of an external standard (50-500 bp, GE Healthcare, USA) and with an in-house amplified internal standard using the Allele Locator 1.03 software (Amersham Biosciences, India).

Data analysis

A total of 51 reproducible bands from 16 SSR primers were scored by length variation as codominant markers for the 27 cultivars and lines studied. The genetic dissimilarities among pineapple individuals were calculated using the methods developed by Bowcock et al. (1994) and Ciampolini et al. (1995), on the basis of pairwise inter-individual comparisons, resulting in a multilocus genetic similarity value complementary to the multilocus genetic distance (Dm) and

Table 1. Pineapple cultivars and lines whose relationships were analyzed.

No.	Varieties/lines	Genetic background	Leaf characters		Fruit characters		Origin
			Spiny/spineless	Color	Skin color	Flesh color	
1	Tainung No. 1	Smooth Cayenne[a] x Yellow skin[b]	Dense spiny	Dark green	Dark yellow	Yellow	Taiwan
2	Tainung No. 2	Smooth Cayenne x Yellow skin	Spineless margins except at the apices	Olive green	Golden yellow	Yellow	Taiwan
3	Tainung No. 3	Yellow skin x Smooth Cayenne	Spineless margins except at the apices	Olive green	Reddish yellow	Yellow	Taiwan
4	Tainung No. 4	Smooth Cayenne x Singapore Spanish[c]	Dense spiny	Green	Golden yellow	Yellowish white	Taiwan
5	Tainung No. 5	Smooth Cayenne x Singapore Spanish	Spiny	Mostly green, some dark red	Golden yellow	Bright yellow	Taiwan
6	Tainung No. 6	Singapore Spanish x Smooth Cayenne	Dense spiny	Mostly green, some dark red	Reddish yellow	Yellow	Taiwan
7	Tainung No. 7	Smooth Cayenne x Yellow skin	Spineless margins except at the apices	Yellowish green	Reddish yellow	Bright yellow	Taiwan
8	Tainung No. 8	Smooth Cayenne x Yellow skin	Spineless margins except at the apices	Yellowish green	Reddish yellow	Bright yellow	Taiwan
9	Tainung No. 11	Smooth Cayenne x Yellow Mouritius	Spineless margins except at the apices	Green	Golden yellow	Bright yellow	Taiwan
10	Tainung No. 13	Smooth Cayenne x Tainung No. 8	Spineless margins except at the apices	Mostly green, some dark red	Dark yellow	Yellow	Taiwan
11	Tainung No. 16	Smooth Cayenne x Rough[b]	Spineless margins except at the apices	Mostly green, some light purple	Yellow	Yellow	Taiwan
12	Tainung No. 17	Smooth Cayenne x Rough	Spineless margins except at the apices	Mostly green, some brownish red	Yellow	Bright yellow	Taiwan
13	Tainung No. 18	Smooth Cayenne x 1A1	Spineless margins except at the apices	Green	Yellow	Yellow	Taiwan
14	Tainung No. 19	Smooth Cayenne x Rough	Spineless margins except at the apices	Dark green	Dark yellow	Yellow	Taiwan
15	Tainung No. 20	Unknown, somatic mutation from unknown clone	Spineless	Dark green	Dark yellow	White	Hawaii, USA
16	Tainung No. 21	(Smooth Cayenne x Tainung No. 4) x (Smooth Cayenne x Rough)	Spineless margins except at the apices	Green	Yellow	Golden yellow	Taiwan
17	Tainung No. 22	Tainung No. 3 x Tainung No. 8	Spineless margins except at the apices	Dark green	Reddish yellow	Bright yellow	Taiwan
18	Line KI-5F	Unknown	Spineless	Green	Yellow	Bright yellow	Taiwan
19	Line KI-16C	Unknown	Spineless	Green	Yellow	Bright yellow	Taiwan
20	Line M4	Unknown	Spineless margins except at the apices	Green	Yellow	Bright yellow	Taiwan
21	Line R5	Unknown	Spineless	Mostly green, some brownish red	Red	Bright yellow	Taiwan
22	Line S4	Unknown	Spineless	Green	Yellow	Bright yellow	Taiwan
23	Line Y7	Unknown	Spineless	Dark green	Green	White	Taiwan
24	Line T1	Unknown	Spineless	Green	Yellow	Bright yellow	Taiwan
25	Line Ma	Unknown	Spiny	Mostly green, some brownish red	Yellowish green	Bright yellow	Malaysia
26	Line Lee	Unknown	Spineless margins except at the apices	Green	Yellow	Bright yellow	Taiwan
27	Line TN4-A	Unknown	Dense spiny	Green	Golden yellow	Yellowish white	Taiwan

[a]Smooth Cayenne belongs to the Cayenne group. [b]Yellow skin and Rough belong to the Queen group. [c]Singapore Spanish belongs to the Spanish group.

modified to the genetic dissimilarity by 1-Dm. Cluster analysis was generated from the pairwise dissimilarity matrix by the unweighted pair-group method (UPGMA) using a molecular evolutionary genetics analysis program (MEGA, version 5.05, Tamura et al., 2011).

RESULTS

Two electrophoresis graphs are shown in Figures 1 and 2. According to the electrophoretograms, either one or two PCR products were observed for each sample. After data analysis for 16 SSR markers, the number of alleles per locus ranged from 2 to 6 (average 3.19), for a total of 51 alleles. The 27 pineapple cultivars and lines could be successfully distinguished from one another by the 16 SSR markers, with two exceptions: 'Line KI-5F' / 'Line KI-16C' and 'Tainung No. 4' / 'Line TN4-A' (Figure 3).

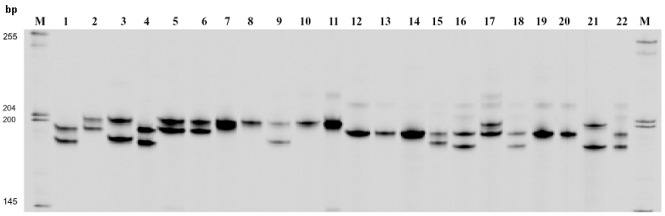

Figure 1. Acom-64.22 simple sequence repeat (SSR) locus analysis of polymorphism in 27 pineapple cultivars and lines. *Lane M* = DNA marker. *Lanes 1-27* = the cultivars and lines listed in Table 1.

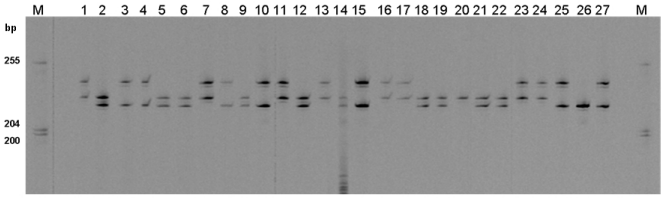

Figure 2. Acom-12.12 simple sequence repeat (SSR) locus analysis of polymorphism in 27 pineapple cultivars and lines. *Lane M* = DNA marker. *Lanes 1-27* = the cultivars and lines listed in Table 1.

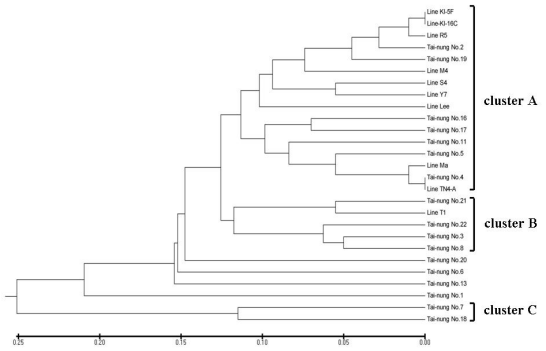

Figure 3. Dendrogram showing the genetic relationships among 27 pineapple cultivars and lines using simple sequence repeat (SSR) markers. The scale bar represents the genetic distance.

Using the SSR molecular markers of 27 pineapple cultivars and lines, all possible pairwise genetic distances were calculated. They ranged from 0 to 0.78 with an average of 0.26 (Table 2). The dendrogram was obtained by UPGMA analysis. Accessions of 'Tainung No. 7' and 'Tainung No. 21' had the greatest genetic distance, 0.78, while the two pairs of accessions, 'Tainung No. 4' / 'Line TN4-A' and 'Line KI-5F' / 'Line KI-16C' exhibited the lowest genetic distance, 0.

Table 2. Genetic distance matrix of 27 pineapple cultivars and lines using 16 simple sequence repeat (SSR) loci.

	P1[a]	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26	P27
P1																											
P2	0.33																										
P3	0.34	0.21																									
P4	0.41	0.18	0.21																								
P5	0.43	0.17	0.30	0.10																							
P6	0.51	0.31	0.32	0.38	0.23																						
P7	0.72	0.65	0.62	0.54	0.60	0.56																					
P8	0.35	0.24	0.10	0.25	0.37	0.42	0.69																				
P9	0.37	0.21	0.37	0.18	0.15	0.23	0.38	0.38																			
P10	0.43	0.22	0.40	0.23	0.30	0.33	0.58	0.44	0.28																		
P11	0.45	0.20	0.35	0.15	0.17	0.33	0.54	0.43	0.26	0.35																	
P12	0.49	0.25	0.35	0.20	0.17	0.28	0.62	0.36	0.26	0.41	0.14																
P13	0.74	0.37	0.49	0.53	0.55	0.42	0.23	0.50	0.42	0.44	0.46	0.57															
P14	0.43	0.12	0.21	0.18	0.15	0.23	0.48	0.24	0.18	0.22	0.28	0.28	0.37														
P15	0.69	0.29	0.33	0.33	0.35	0.32	0.50	0.33	0.33	0.42	0.26	0.44	0.32	0.24													
P16	0.31	0.24	0.22	0.19	0.22	0.33	0.78	0.22	0.31	0.35	0.29	0.21	0.52	0.30	0.49												
P17	0.39	0.26	0.12	0.23	0.29	0.31	0.69	0.13	0.36	0.49	0.25	0.28	0.59	0.26	0.29	0.24											
P18	0.36	0.05	0.15	0.18	0.17	0.20	0.52	0.21	0.18	0.22	0.20	0.28	0.28	0.07	0.18	0.27	0.20										
P19	0.36	0.05	0.15	0.18	0.17	0.20	0.52	0.21	0.18	0.22	0.20	0.28	0.28	0.07	0.18	0.27	0.20	0.00									
P20	0.33	0.11	0.24	0.27	0.29	0.35	0.67	0.25	0.30	0.31	0.23	0.16	0.37	0.21	0.45	0.15	0.24	0.13	0.13								
P21	0.41	0.07	0.18	0.18	0.18	0.23	0.49	0.25	0.18	0.25	0.20	0.29	0.29	0.10	0.21	0.31	0.23	0.02	0.02	0.16							
P22	0.49	0.17	0.20	0.23	0.28	0.28	0.58	0.26	0.20	0.30	0.25	0.36	0.39	0.20	0.21	0.32	0.20	0.12	0.12	0.26	0.15						
P23	0.39	0.21	0.27	0.19	0.29	0.38	0.43	0.33	0.16	0.29	0.26	0.44	0.40	0.21	0.22	0.37	0.24	0.16	0.16	0.30	0.19	0.11					
P24	0.33	0.20	0.23	0.23	0.29	0.28	0.48	0.24	0.18	0.28	0.25	0.31	0.28	0.23	0.21	0.11	0.26	0.17	0.17	0.18	0.21	0.14	0.16				
P25	0.46	0.21	0.19	0.02	0.13	0.29	0.43	0.22	0.16	0.26	0.18	0.23	0.43	0.15	0.25	0.22	0.21	0.15	0.15	0.30	0.16	0.20	0.16	0.21			
P26	0.59	0.16	0.25	0.22	0.30	0.41	0.60	0.22	0.31	0.29	0.42	0.48	0.35	0.19	0.28	0.37	0.43	0.14	0.14	0.34	0.17	0.21	0.28	0.27	0.20		
P27	0.41	0.18	0.21	0.00	0.10	0.38	0.54	0.25	0.18	0.23	0.15	0.20	0.53	0.18	0.33	0.19	0.23	0.18	0.18	0.27	0.18	0.23	0.19	0.23	0.02	0.22	

[a]P1-P27 refer to the pineapple cultivars and lines listed in Table 1.

According to the dendrogram, the 27 pineapple cultivars and lines were clustered into three main clusters and four individual clusters. Cluster A included 'Line KI-5F', 'Line KI-16C', 'Line R5', 'Tainung No. 2', 'Tainung No. 19', 'Line M4', 'Line S4', 'Line Y7', 'Line Lee', 'Tainung No. 16', 'Tainung No. 17', 'Tainung No. 11', 'Tainung No. 5', 'Tainung No. 4', 'Line TN4-A', and 'Line Ma'. Cluster B included 'Tainung No. 21', 'Line T1', 'Tainung No. 22', 'Tainung No. 3', and 'Tainung No. 8'. 'Tainung No. 7' was grouped with 'Tainung No. 18' in cluster C. In addition, 'Tainung No. 20', 'Tainung No. 6', 'Tainung No. 13', and 'Tainung No. 1' belong to individual clusters (Figure 3).

DISCUSSION

Either one or two PCR products derived from SSR markers were observed for each sample, representing sample homogeneity and heterogeneity, respectively. This result is congruent with the chromosome background of pineapple cultivars which are diploid plants (Smith and Downs, 1979). According to the genetic distance derived from SSR markers of 27 pineapple cultivars in the study, the results show that there is a high degree of genetic variation among pineapple cultivars in Taiwan. This variation has also been revealed in DNA studies of pineapple cultivars by AFLP markers (Kato et al., 2005) and SSR markers (Kinsuat and Kumar, 2007). It may result from the combination of self-incompatibility, high levels of somatic mutation, and intraspecific hybridization in pineapple (Kato et al., 2005).

In cluster A, two local lines, 'Line KI-5F' and 'Line KI-16C', had identical genotypes, indicating that they share the same vegetative clone and are separated from the others. The local

line ‘Line TN4-A’ and ‘Tainung No. 4’ also had the same genotype, indicating that the local line is the vegetative clone of ‘Tainung No. 4’. The selected line from Malaya, ‘Line Ma’ was very closely related to ‘Tainung No. 4’. Therefore, we suggest that ‘Line Ma’ originated from ‘Tainung No. 4’ and was selected by bud mutation. In addition, ‘Tainung No. 5’ was closely related to ‘Tainung No. 4,’ according to the DNA markers. The result agrees with the genealogy of both cultivars, which share the same parents (Yang, 1951; Kang, 1953). ‘Tainung No. 16’ and ‘Tainung No. 17’ were also closely related. The result agrees with their breeding history, as they were selected after artificial hybridization using ‘Smooth Cayenne’ as a female parent and ‘Rough’ as a male parent (Chang and Kuan, 2001) (Table 1). Other cultivars of the cluster, including ‘Tainung No. 2’, ‘Tainung No. 11,’ and ‘Tainung No. 19,’ were all selected after artificial hybridization using ‘Smooth Cayenne’ as a female parent and ‘Yellow Skin’ / ‘Yellow Mouritius’/ ‘Rough’ (Queen type) as a male parent (Yang, 1951; Kang, 1953; Chang and Kuan, 2001). The other local lines of cluster A, including ‘Line R5’, ‘Line M4’, ‘Line S4’, ‘Line Y7’, and ‘Line Lee’, can be separated from each of the others. These local lines collected from Taiwan may be further characterized to develop new cultivars. In cluster B, ‘Tainung No. 22’, ‘Tainung No. 3’, and ‘Tainung No. 8’ were very closely related. This result is partially consistent with the breeding history that ‘Tainung No. 22’ was selected after artificial hybridization using ‘Smooth Cayenne’ as a female parent and ‘Tainung No. 8’ as a male parent (Kuan et al., 2012). In cluster C, both ‘Tainung No. 7’ and ‘Tainung No. 18’ were selected from hybridization using ‘Smooth Cayenne’ as a female parent and ‘Yellow skin’ / ‘1A1’ as a male parent (Yang, 1951; Kang, 1953; Chang and Kuan, 2001).

‘Tainung No. 20’ was selected from the bud mutation of unknown clones originating from Hawaii (Lin, 2004). This cultivar has a freshly cut surface that is pale in color, differing from the others, all of which have a freshly cut surface that is a bright or dark color (Table 1). This difference explains why ‘Tainung No. 20’ is separated genetically from the others. ‘Tainung No. 13’ resulted from hybridization using ‘Smooth Cayenne’ as a female parent and ‘Tainung No. 8’ as a male parent (Chang and Kuan, 2001). According to its genealogy, this cultivar did not have a unique genetic background or originate from another country outside Taiwan. Therefore, the unique genotype of ‘Tainung No. 13’ cannot be explained. It might result from the unique direction of selection achieved by the breeder. As for ‘Tainung No. 6,’ the cultivar resulted from artificial hybridization in a different direction, using ‘Smooth Cayenne’ as a male parent and ‘Singapore Spanish’ as a female parent and separated from other cultivars in Taiwan (Yang, 1951; Kang, 1953). This separation produced qualities that place ‘Tainung No. 6’ in an individual cluster.

According to the dendrogram and morphological characters, there is no obvious correlation between the genotype derived from SSR markers and the phenotype, including the color and texture of the leaf surface, the color of the fruit skin, and the color of the freshly cut surface. Similar results were reported by other studies that used DNA marker analysis to classify pineapple cultivars (Kato et al., 2005; Shoda et al., 2012). Collins (1960) showed that whether the outside edge of the leaf is spiny or lack spines is controlled by a single gene. Fruit skin color is controlled by the accumulation of carotenoids (Brat et al., 2004), chlorophyll degradation (Dull, 1971), and the accumulation of anthocyanins (Brat et al., 2004). The variation in accumulation levels of the different pigments produces a range of skin types, including green, yellow, gold, pink, and red (Sanewski, 2011). The color of the freshly cut fruit is controlled by the accumulation of carotenoids (Dull, 1971; Brat et al., 2004), and white, bright yellow, and dark yellow fruit may be characteristic of different pineapple cultivars (Sanewski, 2011). The accumulation of anthocyanins on the leaf surface is a key factor affecting the phenotype of leaf color. The phenomenon is also very popular in other plants with

different functional roles (Gould et al., 1995, 2000). The biosynthesis of those plant pigments can be disrupted by one or a few genes (Harvaux and Kloppstech, 2001). Therefore, a difference in one or a few genes can sometimes significantly change plant morphology (Kato et al., 2005), which can explain why there is no correlation between the molecular data derived from SSR markers and the morphological characters observed.

Data from the present study showed that microsatellite markers were useful for evaluation of genetic diversity of pineapple cultivars and lines. Analysis using SSR markers can offer informative characters to identify pineapple cultivars. The genetic relationships among pineapple cultivars in Taiwan essentially agree with the genealogy of their breeding history. However, there is no obvious correlation between the genetics of SSR markers and the morphological characters displayed by the plants.

Conflicts of interest

The authors declare no conflict of interest.

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