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Genetic Diversity and Sectional Relationships from an Amplified Fragment Length Polymorphism Analysis of Taiwan Bananas

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Phylogenetic relationships among 19 *Musa* species or cultivars were examined through DNA fingerprinting with amplified fragment length polymorphism (AFLP) analysis. The AFLP analysis was performed on the *Musa* species or cultivars with 21 primer combinations, yielding a total of 6,348 DNA bands, among which 6,113 (96.3%) were polymorphic. *M. itinerans* var. *formosana* demonstrated 133 monomorphic bands, which is the most among all samples. Unweighted pair–group method with arithmetic averages was used as a cluster program to divide *M. itinerans*, *M. acuminate*, *M. balbisiana* and 16 cultivars into different major groups. The genetic similarity coefficient among all the species was 0.23. Bananas with the A genome were clustered into the same major group. The similarity coefficients of 'Pei Chiao' with 'Giant Cavendish' (0.99) and 'Dwarf Cavendish' (0.97) indicated that these cultivars share a strong phylogenetic relationship. Within the primer combination of E–TAC/M–GTC, the fingerprint pattern of 'Pei Chiao' was compared with that of 'Giant Cavendish' and 'Dwarf Cavendish' to show two polymorphic bands of 255 and 238 bp. Although 'Pei Chiao' with the AAA genome was highly similar in genetic constitution to 'Giant Cavendish' and 'Dwarf Cavendish', it still can be discriminated from its close relatives according to the information of few polymorphic fragments. In addition, the phylogenetic analysis results revealed that the genetic organization of native Taiwan species, *M. itinerans* var. *formosana*, differed significantly from that of the other banana species. The findings of this study may facilitate germplasm identification and genetic breeding of banana species and cultivars.

Key words: amplified fragment length polymorphism, banana, *Musa*, phylogenetic relationships

INTRODUCTION

Bananas are a staple crop found in tropical and subtropical regions, and their cultivars are typically triploid hybrids (Noyer *et al*., 2000). Triploid bananas originate from the intra– and interspecific hybridization between two diploid species *M. acuminata* Colla (AA) and *M. balbisiana* Colla (BB) (Simmonds & Shepherd, 1955). The descendants of these banana hybrids have evolved various genome compositions including AB, AAA, AAB, ABB, BBB, AAAA, AAAB, AABB, and ABBB (Stover & Simmonds, 1987). Most dessert bananas (AAA) are autoploids and homogenomic hybrids, whereas plantains (AAB) and cooking bananas (ABB) are alloploids and heterogenomic hybrids (Ude *et al*., 2002a). Banana cultivars and landraces of AAA, AAB, and ABB groups are mainly cultivated in Africa, Asia, and Latin America, whereas the cultivars of the Cavendish subgroup (AAA) are produced in Colombia, Costa Rica, and Ecuador (Creste *et al*., 2003).

The main banana cultivar grown in Taiwan is Cavendish banana cv. 'Formosana' (*M. acuminate*, AAA) (Ko *et al*., 2009), commonly named as 'Pei Chiao', and its genetic diversity remains under–explored. Previous studies on wild banana species endemic to Taiwan have mostly discussed their phenotypic characterization; a few have adopted small–scale DNA fingerprinting to explore their genetic diversity (Chiu *et al*., 2004; 2007). Currently, wide research on the genetic diversity of Taiwan's wild banana species and their phylogenetic relationships with other species is scarce. Therefore, more molecular information from analyzing the genetic diversity of Taiwan's wild bananas can clarify their ecological distribution, facilitate their conservation (Wang *et al*., 2007), and guide the preservation and utilization of their genetic resources (Padmesh *et al*., 2012).

DNA molecular diagnostics is widely used in species identification. Several related techniques have been employed to examine genetic diversity in the genus *Musa*. Amplified fragment length polymorphism (AFLP) analysis has been applied in the evaluation of genetic diversity of Omani banana cultivars and various banana species cultivated in different regions (Al–Saady *et al*., 2010), as well as in the identification, classification and diversity analysis of *Musa cultivars* (Bhat *et al*., 2004). Intersimple sequence repeat has been adopted to analyze the genetic variation in the wild *Musa* germplasm and *Musa acuminata* Colla (Lamare & Rao, 2015; Padmesh *et al*., 2012). Random amplified polymorphic DNA (RAPD) was used to identify A and B genome groups in *Musa* L. (Pillay *et al*., 2000) and analyze their genetic variation (Martin *et al*., 2006). Restriction frag-

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Table 1. Plant materials of genus *Musa* used in this study

No.	Accession ID [a]	Species/hybrid	Genome	Common name	Abbreviation
1	00264380	*M. acuminata*	AA w [b]	*M. acuminata*	ACU
2	00105288	*M. acuminata*	AA cv [b]	Sucrier	SUC
3	00105297	*M. a.* × *M. b.*	AB	Ney Poovan	NPO
4	00264577	*M. acuminate* (Triploid)	AAA	Yangambi KM5	YAN
5	00105304	*M. balbisiana*	BB w [c]	*M. balbisiana*	BAL
6	00264844	*M. a.* × *M. b.* (Triploid)	ABB	Monkey	MON
7	00105671	*M. acuminata* (Triploid)	AAA	Morado	MOR
8	00105466	*M. acuminata* (Triploid)	AAA	Giant Cavendish	GCA
9	00105411	*M. acuminata* (Triploid)	AAA	Dwarf Cavendish	DCA
10	00106025	*M. acuminata* (Triploid)	AAA	Pei Chiao	PCH
11	00106043	*M. a.* × *M. b.* (Triploid)	AAB	Assam	ASS
12	00264700	*M. a.* × *M. b.* (Triploid)	AAB	Rilian	RIL
13	00264835	*M. a.* × *M. b.* (Triploid)	ABB	Ice Cream	ICR
14	00264335	*M. a.* × *M. b.* (Triploid)	ABB	Pelipita	PEL
15	00264826	*M. balbisiana* (Triploid)	BBB	Cooking	COO
16	00264853	*M. balbisiana* (Triploid)	ABB	Nibah	NIB
17	00264522	*M. balbisiana* (Tetraploid)	AAAA	Fhia–17	F–17
18	00264497	*M. a.* × *M. b.* (Tetraploid)	AAAB	Fhia–01	F–01
19	00105242	*M. itinerans*	unknown	Formosana	FOR

[a] Accession ID was based on the National Plant Genetic Resources Center of Taiwan Agricultural Research Institute.
[b] *M. acuminate AAw* wild type, *AAcv* cultivar
[c] *M. balbisiana BBw* wild type

Table 2. AFLP DNA fragments obtained for 19 *Musa* species or cultivars based on 21 pairs of primers

Primer Pair	Primer combination		Total No. of bands	Polymorphic bands	Monomorphic bands	Polymorphism (%)
	*Eco*RI	*Mse*I				
1	AGA	CAA	337	322	15	95.5
2	AGA	CAC	260	246	14	94.6
3	AGA	CTT	268	259	9	96.6
4	AAG	CAT	378	364	14	96.3
5	AAG	CTA	124	118	6	95.2
6	AAC	CAC	214	204	10	95.3
7	ACA	CTA	262	253	9	96.6
8	ACA	CTT	187	178	9	95.2
9	ACT	CAA	220	206	14	93.6
10	ACT	CAC	177	166	11	93.8
11	ACT	CTA	113	103	10	91.2
12	ACT	CTT	258	252	6	97.7
13	TGA	GGT	455	444	11	97.6
14	TGA	GTG	371	362	9	97.6
15	TGA	GCT	430	417	13	97.0
16	TAG	GGT	483	465	18	96.3
17	TAG	GAG	313	297	16	94.9
18	TAG	GTC	544	536	8	98.5
19	TAC	GTG	344	333	11	96.8
20	TCG	GAG	277	269	8	97.1
21	TCA	GGT	333	319	14	95.8
Total for all primers			6348	6113	235	96.3

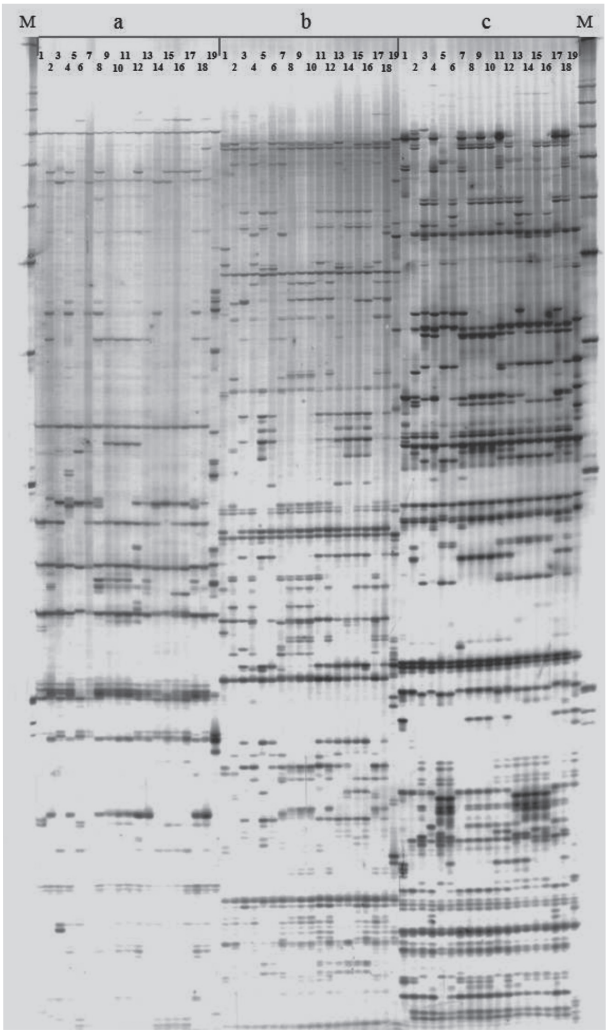

Fig. 1. AFLP DNA profiling generated by three primer combinations a. (E–AGA/M–CAC); b. (E–ACA/M–CTA); c. (E–ACT/M–CTT). Numbers of lanes for the banana species are listed in Table 1.

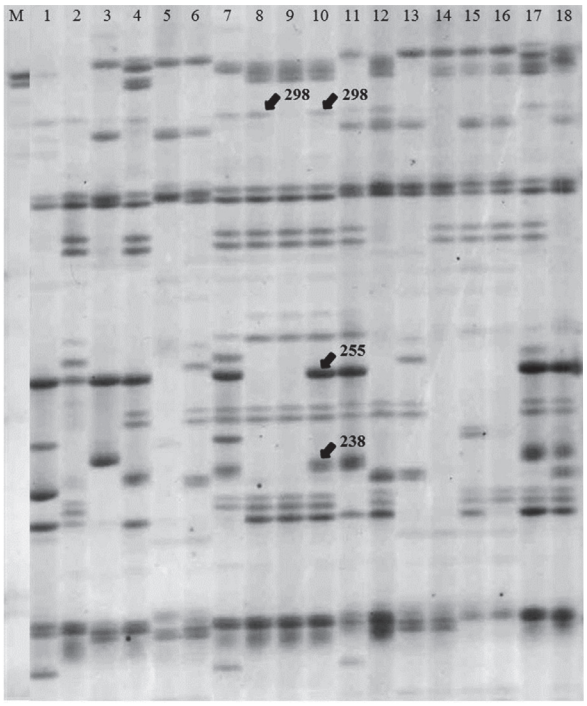

Fig. 2. AFLP profiling generated by a primer combination of E–TAC/M–GTC. Numbers of lanes for the banana species are listed in Table 1.

ment length polymorphism (RFLP) was used to determine maternal and paternal lineages within *Musa* (Carreel *et al*., 2002). The restriction–site variations in the internal transcribed spacers of nuclear ribosomal RNA genes were analyzed to discriminate the A and B genomes of *Musa* (Nwakanma *et al*., 2003). Sequence–related amplified polymorphism was used to analyze the *Musa* genetic diversity (Youssef *et al*., 2011).

The phenotype of banana species and cultivars can vary depending on the environmental conditions, and the phenotypic variation affects the accuracy of identifying the characteristics or traits. Molecular diversity analysis can be conducted on banana DNA samples to prevent the influence of environmental factors. AFLP is a molecular diagnostic type of DNA detection technique that facilitates phylogenetic analysis. It combines restriction–based fingerprinting with polymerase chain reaction (PCR)–based marker techniques (Vos *et al*., 1995; Ude *et al*., 2002a, 2002b), enabling repeatability in experimental results and generating numerous genome–level polymorphic bands. Because of these advantages, AFLP can be used to determine the genetic relationships among different banana cultivars (Loh *et al*., 2000) and identify genomes within banana cultivars and hybrids (Wongniam *et al*., 2010). Thus, AFLP is efficient for examining the genetic diversity of bananas for classification (Opara *et al*., 2010). On the basis of the aforementioned studies, the present study adopted AFLP to analyze the genetic diversity of diverse cultivars and wild species of banana endemic to Taiwan for determining their phylogenetic relationships.

MATERIALS AND METHODS

Plant materials

This study used 19 *Musa* species and cultivars (two *M. acuminata* materials, two *M. balbisiana* materials, 14 intra– or interspecific hybrids of *M. acuminata* and *M. balbisiana*, and one species of *M. itinerans* var. *formosana*) cultivated in a banana germplasm garden managed by the Taiwan Agricultural Research Institute (Chiayi branch) (Table 1).

Genomic DNA extraction

Young cigar leaves were obtained from all the banana samples. On the basis of the method proposed by Doyle and Doyle (1990), DNA was isolated from the leaves, purified, and diluted at a final concentration of 100 ng/μL, and stored at –20°C. The DNA was used as the template for the PCR reactions.

AFLP procedure

The AFLP process was conducted in the following steps (Vos *et al*., 1995): Restriction digestion of genomic DNA with *Eco*RI and *Mse*I was conducted at 37°C for 4

hours. Subsequently, the enzymes were inactivated at 70°C for 20 min. The digested DNA products were ligated to both *Eco*RI and *Mse*I adapters overnight at 16°C to produce template DNA for preamplification. PCR preamplification was conducted using AFLP primers containing one selective nucleotide. The PCR preamplified products were diluted eightfold in sterile water and used as templates for selective amplification by using AFLP primers containing three selective nucleotides. Table 2 shows the primer and adapter sequences. The PCR products from selective amplification were subjected to 4% denaturing polyacrylamide gel electrophoresis in 1X TBE buffer. DNA fragments on gels were visualized using silver nitrate staining protocol (Bassam *et al*., 1991). The polymorphic fragments were collected and used for further phylogenetic analysis.

Data analysis

The presence of polymorphic bands was scored as 1, and no band was scored as 0. The genetic similarity of the samples was estimated following the method proposed by Nei and Li (1979). Polymorphic data on the samples were processed using NTSYSpc 2.0 to calculate their genetic similarity and subsequently using an unweighted pair–group method with arithmetic average (UPGMA) cluster analysis to plot a phylogenetic dendrogram. Finally, a principal coordinate analysis (PCoA) was conducted to present the distribution of all sample dimensions in a scatter–plot pattern (Ude *et al*., 2002a)

RESULTS

AFLP analysis of the Musa accessions

An analysis of the polymorphisms in 19 *Musa* accessions was performed using 21 AFLP primer combinations. A total of 6,348 DNA bands, ranging from the size of 40 to 1,100 bp in AFLP–based fingerprinting images, were obtained. Among them, 6,113 (96.3%) were polymorphic. The polymorphic variation among the primer combinations was between 91.2% and 98.5%. Of all the primer combinations, E–TAG/M–GTC exhibited the highest proportion of polymorphic bands (98.5%), whereas E–ACT/M–CTA revealed the lowest proportion (91.2%) (Table 2).

Moreover, the number of unique polymorphic bands quietly differed among 13 of the *Musa* accessions (Table 3). According to the fingerprint results, *M. acuminate* had 36 of these bands; 'Sucrier' and 'Yangambi KM5' had 16 and 15, respectively. *M. itinerans* var. *formosana* had 133 unique bands, the largest number of these bands; in the primer combinations of E–AGA/M–CAC, E–ACA/M–CTA, and E–ACT/M–CTT (Fig. 1), it had 10, 7, and 3 specific bands, respectively. 'Giant Cavendish' exhibited only one specific band in the E–ACT/M–CTA combination, as did 'Nibah' in the E–TCA/M–GGT; however, these two banana species had no unique band in the other primer

Table 3. Unique AFLP markers and total number of markers characterizing *Musa* genotypes, represented by the number of specific bands per category

Category	Primer combinations [a]																					Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
M. acuminata	4	–	3	6	–	1	2	–	3	2	4	1	–	–	1	3	3	1	1	–	1	36
Sucrier	2	–	–	1	1	1	–	–	1	1	1	2	2	2	–	–	1	1	–	–	–	16
Ney Poovan	–	–	1	–	–	–	–	–	2	–	1	–	1	–	1	–	–	–	–	–	1	7
Yangambi KM5	–	1	–	–	–	–	–	–	–	3	1	–	1	1	2	1	2	–	–	1	2	15
M. balbisiana	–	–	–	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	2	–	4
Monkey	–	–	–	–	–	1	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	2
Morado	1	1	–	1	–	–	–	–	–	–	–	–	1	1	–	–	–	–	–	–	–	5
Giant Cavendish	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	1
Dwarf Cavendish	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Pei Chiao	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Assam	–	1	–	–	–	1	–	–	–	–	–	–	1	–	–	1	–	1	–	–	–	5
Rilian	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Ice Cream	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Pelipita	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
Cooking	–	–	–	–	–	1	–	1	–	–	–	–	–	–	–	1	1	–	–	–	–	4
Nibah	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1
Fhia–17	–	–	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	3
Fhia–01	–	1	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	3
Formosana	8	10	4	5	4	5	7	8	7	2	2	3	5	5	9	12	9	5	10	5	8	133
Total	15	14	9	14	6	10	9	9	14	11	10	6	11	9	13	18	16	8	11	8	14	235

[a] Numbers of primer combinations listed in Table 2

combinations. Peculiarly, 'Pei Chiao', 'Dwarf Cavendish', 'Rilian', 'Ice Cream', and 'Pelipita' revealed no specific bands. 'Pei Chiao' and 'Giant Cavendish' shared a 298–bp band in the E–TAC/M–GTC. 'Pei Chiao' contained two bands of 255 and 238 bp, whereas 'Giant Cavendish' and 'Dwarf Cavendish' did not (Fig. 2).

Genetic diversity analysis

In the UPGMA cluster analysis, *M. itinerans* var. *formosana*, *M. acuminata*, *M. balbisiana*, and hybrids were divided into different major groups, which were differentiated in a significant variation of genetic distance and had a similarity coefficient of 0.23. Except for *M. itinerans* var. *formosana*, the remaining 18 *Musa* species (*M. acuminata*, *M. balbisiana*, and hybrids) had genetic similarity coefficients ranging from 0.45 to 0.99 and were therefore clustered into two major groups: the first and second. The first major group can be diversified into two subgroups: a subgroup comprising *M. balbisiana*, 'Cooking', 'Nibah', and 'Pelipita', with a genetic similarity coefficient of 0.66, and the alternative, comprising 'Ice Cream', 'Monkey', and 'Ney Poovan', with a genetic similarity coefficient of 0.71. The second major group encompassed three subgroups: the first group comprised 'Fhia–01', 'Rilian', and 'Assam', with a genetic similarity coefficient of 0.72; the second comprised 'Fhia–17', 'Dwarf Cavendish', 'Pei Chiao', 'Giant Cavendish', 'Morado', 'Yangambi KM5', and 'Sucrier', with a genetic similarity coefficient of 0.71 (notably, 'Pei Chiao' respectively shared a genetic similarity coefficient of 0.99 and 0.97 with 'Giant Cavendish' and 'Dwarf Cavendish', indicating a strong phylogenetic relationship among the three species); the third comprised *M. acuminata*, which shared a genetic similarity coefficient of 0.47 with the aforementioned two subgroups (Fig. 3).

In the second major group, the second and third subgroups belonged to the A genome group (Fig. 3). This major group contained AAA triploids ('Dwarf Cavendish', 'Pei Chiao', 'Giant Cavendish', 'Morado', and 'Yangambi KM5'), an AAAA teraploid ('Fhia–17'), and an AA diploid ('Sucrier'). All of them had a genetic similarity coefficient of 0.70 (Fig. 3).

The PCoA analysis divided the 18 *Musa* species (*M. acuminata*, *M. balbisiana*, and hybrids) into dimension clusters of A, B, and C (Fig. 4). Cluster A comprised banana species with AA, AAA, and AAAA accessions

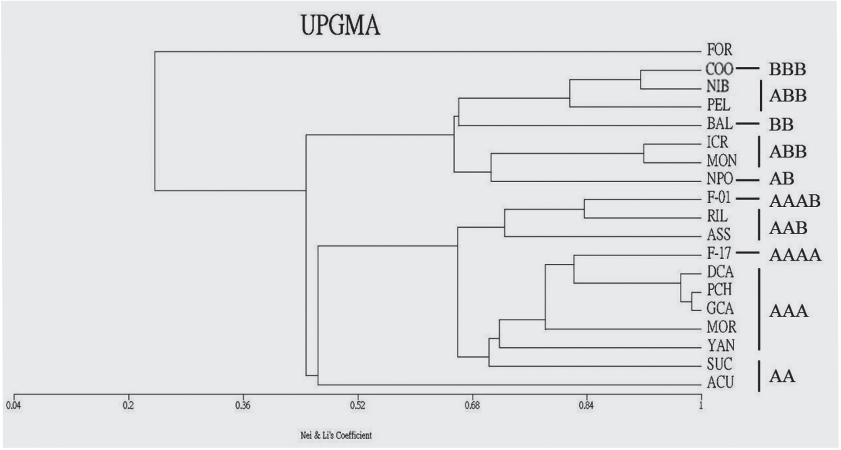

Fig. 3. Dendrogram of genetic similarities in *Musa*, obtained through the AFLP-UPGMA cluster analysis.

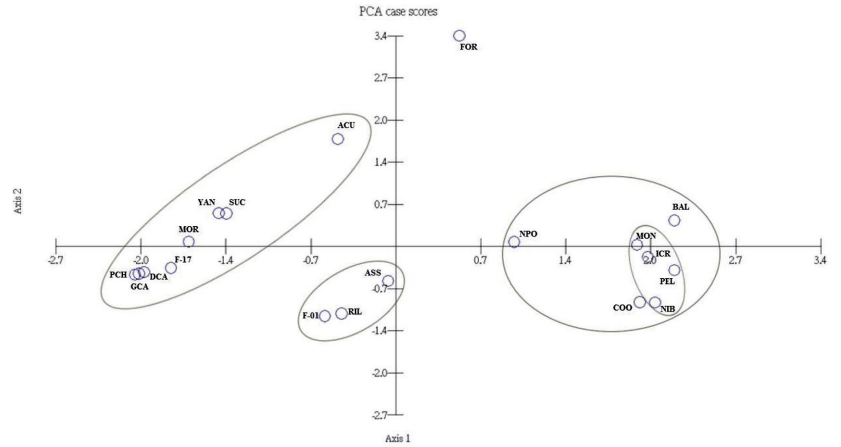

Fig. 4. Scatter plot of the principal coordinate analysis of the 19 *Musa* accessions, derived from the AFLP molecular data.

(e.g., *M. acuminata*, 'Sucrier', 'Yangambi KM5', 'Morado', 'Pei Chiao', 'Giant Cavendish', 'Dwarf Cavendish', and 'Fhia–17'); in particular, the relative distance among 'Pei Chiao', 'Giant Cavendish', and 'Dwarf Cavendish' was close. Cluster B comprised banana species with AAB and AAAB accessions ('Assam', 'Rilian', and 'Fhia–01'). Cluster C comprised banana species with AB, ABB, BB, and BBB accessions (e.g., *M. balbisiana*, 'Ney Poovan', 'Monkey', 'Ice Cream', 'Pelipita', 'Nibah', and 'Cooking'); in particular, the dimension distributions of 'Monkey', 'Ice Cream', 'Pelipita', and 'Nibah' with the same genome of ABB were close to one another (Fig. 4).

DISCUSSION

This study used the AFLP method to examine the phylogenetic relationships among 19 *Musa* accessions. In the phylogenetic analysis results, Cavendish bananas, 'Pei Chiao', 'Giant Cavendish', and 'Dwarf Cavendish' had a strong relationship and were closely related to 'Morado', 'Yangambi KM5', 'Sucrier', and 'Fhia–1'. These banana species, with the A genome constitution, were clustered into the same subgroup (Fig. 4). Ude *et al*. (2002a, 2002b) adopted AFLP to perform polymorphic analysis and classify *M. acuminata* (AA) and dessert bananas (AAA) into the same subgroup, suggesting a highly similar lineage relationship of dessert bananas with cultivars and wild species (AA). In accordance with a previous study that banana accessions with BB, ABB, and AAB genomes can be divided into different subgroups, our study clustered banana species with AB, BB, ABB, AAB, BBB, and AAAB genomes into different subgroups. Moreover, compared with RAPD, the AFLP analysis yielded more polymorphic data and was more effective in identifying the genetic differences among plantain accessions (Ude *et al*., 2003).

The results of the AFLP analysis indicated that 'Pei Chiao', a major banana cultivar in Taiwan (Ko *et al*., 2009), had a close phylogenetic relationship with 'Giant Cavendish'. These two species demonstrated slight genetic diversity and a similarity coefficient of 0.99, and their similarity coefficient with 'Dwarf Cavendish' was 0.97 (Fig. 3). These Cavendish varieties belong to the AAA genome group, and their phylogenetic relationship has been identified in previous studies (De Langhe, 2002). In addition, 'Pei Chiao' and 'Giant Cavendish' possessed a unique band of 298 bp in the E–TAC/M–GTC combination. 'Pei Chiao' also had specific bands of 255 and 238 bp in the aforementioned primer combination, whereas 'Giant Cavendish' and 'Dwarf Cavendish' did not; such differences in banding patterns can serve as molecular markers for cultivar identification in the Cavendish varieties (Fig. 2). For example, Loh *et al*. (2008) conducted an AFLP analysis of 8 primer combinations on 16 banana cultivars to generate 555 polymorphic bands and 58 unique bands, the latter of which can be used to distinguish between these cultivars.

The AFLP analysis of 21 primer combinations on all the sampled banana species revealed that *M. itinerans* var. *formosana* contained 133 unique polymorphic bands (Fig. 1), and *M. acuminata* had 36 (Table 3). These unique bands can be applied in genetic diversity analyses. As compared with Wong *et al*. (2002), who divided *M. acuminata* and *M. balbisiana* into one phylogenetic cluster and *M. itinerans* into another cluster, the present study further divided *M. itinerans*, *M. acuminata*, and *M. balbisiana* into different clusters and indicated greater genetic differences between *M. itinerans* and the other two species (Fig. 3). Compared with Chiu *et al*. (2007), who compared the polymorphism patterns of *M. itinerans* var. *formosana*, *M. acuminata* ssp. *microcarpa*, *M. acuminata* ssp. *Malaccensis*, and *M. balbisianabased* based on 13 simple–sequence–repeat primer pairs, obtaining a genetic similarity coefficient of 0.57 for these banana species, the present study examined the genetic differences between *M. itinerans* var. *formosana* and the other 18 species (*M. acuminata*, *M. balbisiana*, and hybrids) through AFLP analysis and determined that *M. itinerans* var. *formosana* was an isolated major group and had a genetic similarity coefficient of 0.23 with the other species (Fig. 3), indicating a wide phylogenetic difference.

In summary, this study showed that AFLP–based DNA fingerprinting has high sensitivity and stability, rendering it a suitable method for the phylogenetic analysis of the cultivars and wild species of *Musa*. It can be used to differentiate some phylogenetically related species in *Musa* and examine the genetic differences of *M. itinerans* var. *formosana* from *M. acuminata* and *M. balbisiana* accessions. *M. itinerans* var. *formosana* was also found to exhibit a distinctive molecular pattern and substantial polymorphic differences from the other 18 banana species. Further research should be conducted to investigate the genomic distinctiveness of *M. itinerans* var. *formosana*. The findings regarding the phylogenetic relationships among banana cultivars and wild species in Taiwan can clarify the ecological investigation, germplasm identification, and breed improvement of bananas.

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