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Genetic Diversity in *Musa acuminata* Colla and *Musa balbisiana* Colla and some of their natural hybrids using AFLP Markers

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Abstract Genetic diversity and relationships were assessed in 28 accessions of *Musa acuminata* (AA) Colla and *Musa balbisiana* (BB) Colla, and some of their natural hybrids, using the amplified fragment length polymorphisms (AFLP) technique. Fifteen AFLP +3 primer pairs produced 527 polymorphic bands among the accessions. Neighbor-joining and principal co-ordinate (PCO) analyses using Jaccard's similarity coefficient produced four major clusters that closely corresponded with the genome composition of the accessions (AA, BB, AAB and ABB). The AFLP data distinguished between the wild diploid accessions and suggested new subspecies relationships in the *M. acuminata* complex that are different from those based on morphological data. The data suggested that there are three subspecies within the *M. acuminata* complex (ssp. *burmannica* Simmonds, *malaccensis* Simmonds, and *microcarpa* Simmonds). 'Tjau Lagada' (ssp. *microcarpa*), 'Truncata' [ssp *truncata* (Ridl.) Shepherd] and 'SF247' [ssp. *banksii* (F.Muell) Simmonds] clustered very closely with 'Gros Michel' and 'Km 5', indicating that more than one *M. acuminata* subspecies may be involved in the origin of triploid AAA bananas. 'Calcutta 4' (ssp. *burmannicoides* De Langhe & Devreux) and 'Long Tavoy' (ssp. *burmannica*) were closely related and could be together in the same subspecies. This study also showed that there is much more genetic diversity within *M. balbisiana* that was split into two groups: (1) 'I-63' and 'HND' and (2) 'Los Banos', 'MPL' (Montpellier), '10852', 'Singapuri', 'Etikehel', and 'Butohan 1' as the other.

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Introduction

Musa acuminata and *Musa balbisiana* are the wild diploid ancestors and donors of the A and B genomes of modern bananas, respectively (Simmonds 1962). Current breeding efforts for the improvement of bananas rely on introgressing useful genes from the wild and cultivated diploid progenitors (Ortiz and Vuylsteke 1994). Therefore there is a need to study genetic diversity and relationships among the wild and cultivated diploid accessions and their polyploid relatives.

M. acuminata is a complex of subspecies that have been grouped in several ways by taxonomists. For example, Cheesman (1947, 1948) distinguished four *M. acuminata* forms, based on morphological and geographical criteria: 'Selangor', 'Tavoy', 'Annam' and 'Buitenzorg'. Another form, *M. acuminata* 'Cameron' was later described by Simmonds (1956). Subsequently, a new classification based on morphological characterization and hybridization studies was proposed (Simmonds 1962) whereby the *M. acuminata* (*M.a.*) forms were raised to subspecies status. For instance, 'Selangor' was raised to *M. acuminata* ssp. *malaccensis*, 'Tavoy' to *M.a.* ssp. *burmannica*,', 'Annam' to *M.a.* ssp. *siamea* and 'Cameron' to *M.a.* ssp. *microcarpa*. There is still no consensus among *Musa* researchers on the number of subspecies that exist in the *M. acuminata complex*. Currently, the following subspecies are recognized by some researchers: *banksii*, *burmannica*, *burmannicoides*, *errans* Allen, *malaccensis*, *microcarpa*, *siamea* Simmonds, *truncata and zebrina* nom. Nud. (De Langhe and Devreux 1960; Shepherd 1988; Tezenas du Montcel 1988). However, many issues still remain unresolved including the separation of: (1) ssp. *burmannicoides* from ssp. *burmannica*, (2) ssp. *truncata* from ssp. *microcarpa*, and (3) ssp. *zebrina* from ssp. *malaccensis* (Shepherd 1988; Tezenas

du Montcel 1988). These problems arise largely due to the complete reliance on morphological and cytological characteristics, and perhaps the use of different plant material by different researchers.

The genetic diversity of *M. balbisiana* has not been studied in any great detail despite its valuable agronomic traits such as resistance to diseases and ability to thrive well in drier conditions than *M. acuminata* (Sotto and Rabara 2000). Morphological studies of 105 accessions of *M. balbisiana* in the Philippines have shown a wide genetic variation (Sotto and Rabara 2000). However, no subspecies categories have been designated. New attempts are required to resolve *Musa* taxonomy.

Isozymes and flavonoids have been used to identify and establish relationships among *M. acuminata* subspecies (Jarret and Litz 1986; Horry and Jay 1988). However, these compounds cannot be widely used for the classification of banana because they are expressed only in certain tissues and depend on the developmental stage of the plant, making data collection and comparison between laboratories cumbersome. Recently, DNA markers that are more abundant than morphological characters and free from environmental influence have been used in genetic diversity studies of plants including *Musa* (Jarret et al. 1993; Kaemmer et al. 1997; Crouch et al. 1998a, b; Grapin et al. 1998; Tenkouano et al. 1999; Pillay et al. 2001). Restriction fragment length polymorphism (RFLP) has been particularly useful in *Musa* diversity studies, but this technique is expensive and technically very demanding (Crouch et al. 1998a). Therefore, *Musa* researchers have concentrated on the application of PCR-based markers such as random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs), and amplified fragment length polymorphism (AFLP) for genetic analysis.

Among DNA markers, the amplified fragment length polymorphism (AFLP) technique is being widely used for genetic diversity studies because it reveals significant polymorphism and is a reliable and robust genetic molecular-marker assay. AFLP has been used to discriminate between accessions of a number of plant species including soybean (Maughan et al. 1996), sunflower (Hongtrakul et al. 1997) and barley (Schut et al. 1999). Crouch et al. (1998a) suggested that AFLP technology has a high potential in contributing to the understanding of *Musa* genetics, but there has been only limited application of this technique for germplasm analysis in *Musa* (Engelborghs and Swennen 1999). Therefore, this study was undertaken to: (1) assess genetic diversity and relationships among accessions of *M. acuminata* and *M. balbisiana*, and (2) to identify relationships between the infra-specific categories of *M. acuminata* and the cultivated edible bananas.

Materials and methods

Plant materials

Twenty eight accessions consisting of 12 *M. acuminata*, eight *M. balbisiana* and eight triploid natural intra- or inter-specific hybrids of *M. acuminata* and *M. balbisiana* (Table 1) from the *Musa* gene bank of the International Institute of Tropical Agriculture were used for the study.

Table 1 Accessions from the section *Eumusa* of the genus *Musa*

S/no.	Species/hybrids	Subspecies	Genome	Name
1	*Musa acuminata*	ssp. *microcarpa* Simmonds	AAw[a]	Borneo
2	*Musa acuminata*	ssp. *burmannicoides* De Langhe & Devreux	AAw	Calcutta 4
3	*Musa acuminata*	ssp. *malaccensis* Simmonds	AAw	Selangor
4	*Musa acuminata*	ssp. *burmannica* Simmonds	AAw	Long Tavoy
5	*Musa acuminata*	ssp. *malaccensis*	AAw	Malaccensis Holotype
6	*Musa acuminata*	ssp. *microcarpa*	AAcv[a]	Tjau Lagada
7	*Musa acuminata*	ssp. *malaccensis*	AAcv	Pisang Lilin
8	*Musa acuminata*	ssp. *banksii* (F.Muell) Simmonds	AAcv	SF247
9	*Musa acuminata*	ssp. *banksii*	AAw	Madang
10	*Musa acuminata*	–	AAcv	Pisang Jari Buaya
11	*Musa acuminata*	ssp.*truncata* (Ridl.) Shepherd	AAw	Truncata
12	*Musa acuminata*	ssp. *zebrina nom. Nud*.	AAw	Zebrina
13	*Musa balbisiana*	–	BB	I-63
14	*Musa balbisiana*	–	BB	Honduras (HND)
15	*Musa balbisiana*	–	BB	Montpellier (MPL)
16	*Musa balbisiana*	–	BB	Singapuri
17	*Musa balbisiana*	–	BB	Los Banos
18	*Musa balbisiana*	–	BB	10852
19	*Musa balbisiana*	–	BB	Butohan1
20	*Musa balbisiana*	–	BB	Etikehel
21	Dessert Banana	–	AAA	Gros Michel
22	Dessert Banana	–	AAA	Yangambi Km 5
23	Plantains	–	AAB	Agbagba
24	Plantains	–	AAB	Batard
25	Plantains	–	AAB	Obino l'Ewai
26	Plantains	–	AAB	Asamiensa
27	Cooking banana	–	ABB	Bluggoe
28	Cooking banana	–	ABB	Fougamou

[a] *M. acuminata* accession AAw = wild-type, AAcv = cultivar

DNA isolation

Genomic DNA was extracted from the cigar leaf (youngest unfurled leaf) of the accessions studied and quantified according to Ude et al. (2002).

AFLP procedure

The AFLP procedure involving DNA digestion and ligation, preamp and selective amplification, and silver nitrate staining was done as reported by Ude et al. (2002). Fifteen primer pairs (*Eco*RI+3 and *Mse*I+3) from the GIBCO BRL commercial AFLP kit were used for the selective amplification. Associated PCR temperature cycles and polyacrylamide gel electrophoresis were done according to Lin et al. (1996). The gel was then rinsed with distilled water, air dried and the polymorphic bands were scored over a light box.

Data scoring and analysis

A band was considered polymorphic if it was present in at least one genotype and absent in the others. A matrix was generated in which each band was scored as ‘‘1” if present and as ‘‘0” if absent for each genotype. The NTSYS-pc software package version 2.02f (Rohlf 1998) was used for data analysis. A genetic similarity matrix was computed according to Jaccard’s similarity index (Jaccard 1908). Genetic distances (GD%) were calculated by subtracting the similarity indices from 1 and multiplying the result by 100. Neighbor-joining (Kim et al. 1992) and multidimensional principal co-ordinate analyses (PCO) were used to show relationships among the 28 accessions. PCO analysis was also used to elucidate, in more detail, the relationships among the *M. acuminata* and *M. balbisiana* accessions.

Results

A total of 527 polymorphic bands were scored which ranged from 22 to 109 with a mean of 35 bands per primer pair. The average genetic distance (AGD) between the 28 accessions was 61.3%. The AGD among the *M. acuminata* diploids was 56.2% while that within *M. balbisiana* was 29.5%.

Neighbor-joining (Fig. 1) and PCO analyses (Fig. 2) separated the 28 *Musa* genotypes into four large clusters that corresponded with the genome designation of the plants (AA, BB, AAB and ABB). The first PCO axis (Prin 1) discriminated the *M. acuminata* and *M. balbisiana* accessions, while the second axis (Prin 2) separated the BB, ABB (cooking banana) and AAB (plantain) accessions. Four genetic subgroups (Fig. 1) were recognized within the *M. acuminata* accessions. The first subgroup consisted of ‘Borneo’ (ssp. *microcarpa*) and ‘Selangor’ (ssp. *malaccensis*). The second subgroup comprised ‘Malaccensis Holotype’ (ssp. *malaccensis*), ‘Madang’ (ssp. *banksii*), ‘Pisang lilin’ (ssp. *malaccensis*) and ‘Zebrina’ (ssp. *zebrina*). The third subgroup was made of the dessert bananas (‘Gros Michel’ and ‘Yangambi Km 5’), ‘Tjau Lagada’ (ssp. *microcarpa*), ‘SF247’ (ssp. *banksii*) and ‘Truncata’ (ssp. *truncata*). The accessions ‘Calcutta 4’ (ssp. *burmannicoides*) and ‘Long Tavoy’ (ssp. *burmannica*) formed the fourth subgroup. ‘Pisang jari buaya’ did not group closely with the other *M acuminata* accessions.

PCO analysis of the *M. acuminata* accessions (Fig. 3)showed three subgroups denoted as A, B and C. The A subgroup consisted of: ‘Borneo’, ‘Selangor’, ‘Tjau Lagada’, ‘Truncata’, ‘SF247’, ‘Gros Michel’ and ‘Yangambi Km 5’. The accession ‘Borneo’ was closely related to ‘Selangor’ while ‘Tjau Lagada’ and ‘Truncata’ paired closely. These accessions grouped relatively closely with ‘SF247’. The B supgroup comprised ‘Zebrina’, ‘Madang’, ‘Pisang lilin’ and the ‘Malaccensis holotype’. The ‘Zebrina’ and ‘Malaccensis holo-

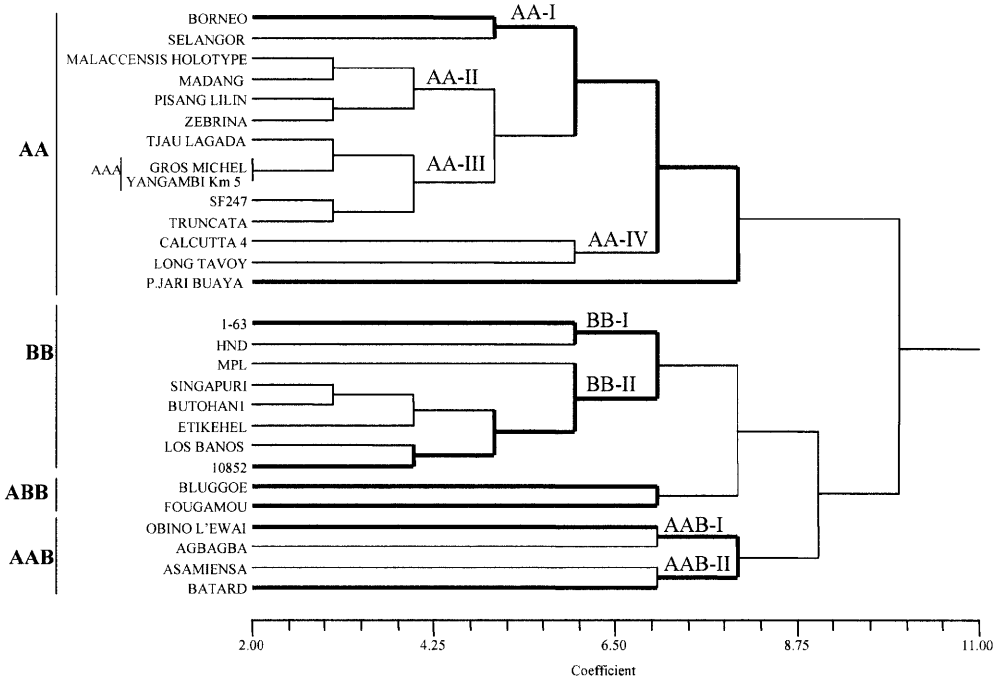

Fig. 1 Dendrogram of 28 *Eumusa* accessions based on Jaccard’s similarity coefficients calculated with 527 AFLP polymorphic bands. Four genomic groups (AA, BB, ABB and AAB) were identified with subgroups within them: *M. acuminata* - AA-I, AA-II, AA-III and AA-IV; *M. balbisiana* – BB-I and BB-II; cooking banana – ABB; plantains – AAB-I and AAB-II

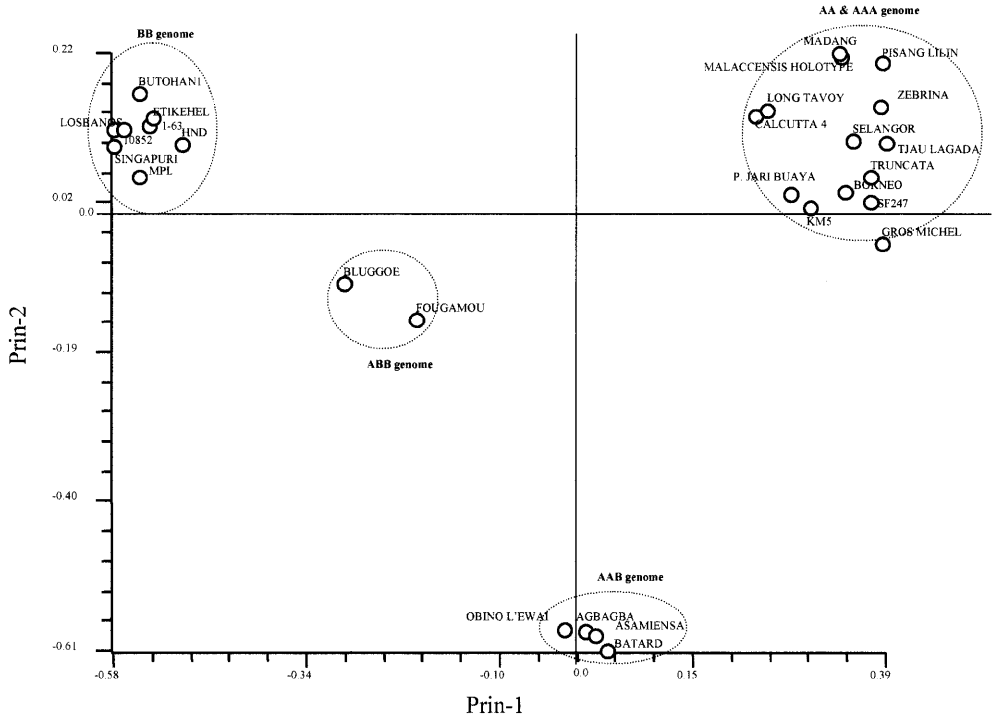

Fig. 2 Principal coordinate map of the 28 accessions from the *Eumusa* section using 527 polymorphic bands derived with 15 AFLP+3 primer pairs. The clusters corresponded to the genomes studied: (*1*) *M. acuminata* (AA and AAA); (*2*) *M. balbisiana* (BB); (*3*) plantains (AAB); and (*4*) cooking bananas (ABB)

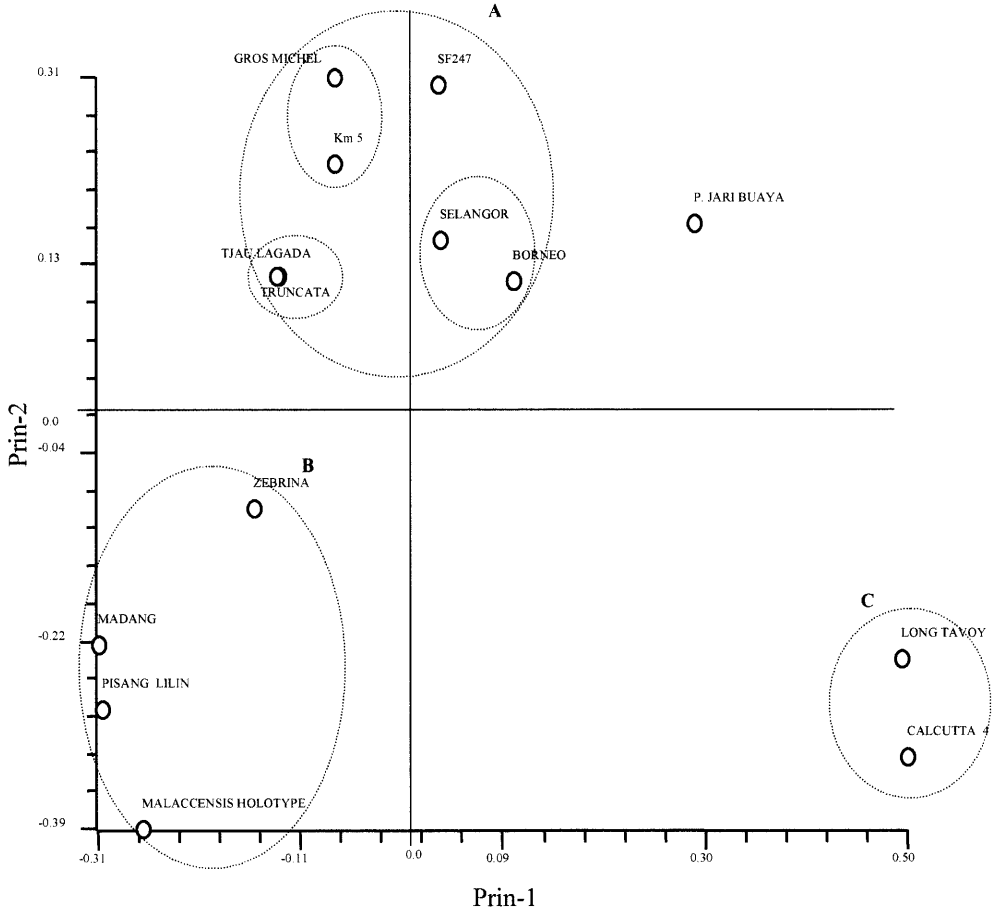

Fig. 3 Principal coordinate map of the *M. acuminata* accessions. Three subgroups were identified with each subgroup represented by a dominant subspecies: *A* = ssp. *microcarpa* type; *B* = ssp. *malaccensis* type; *C* = ssp. *burmannica* type

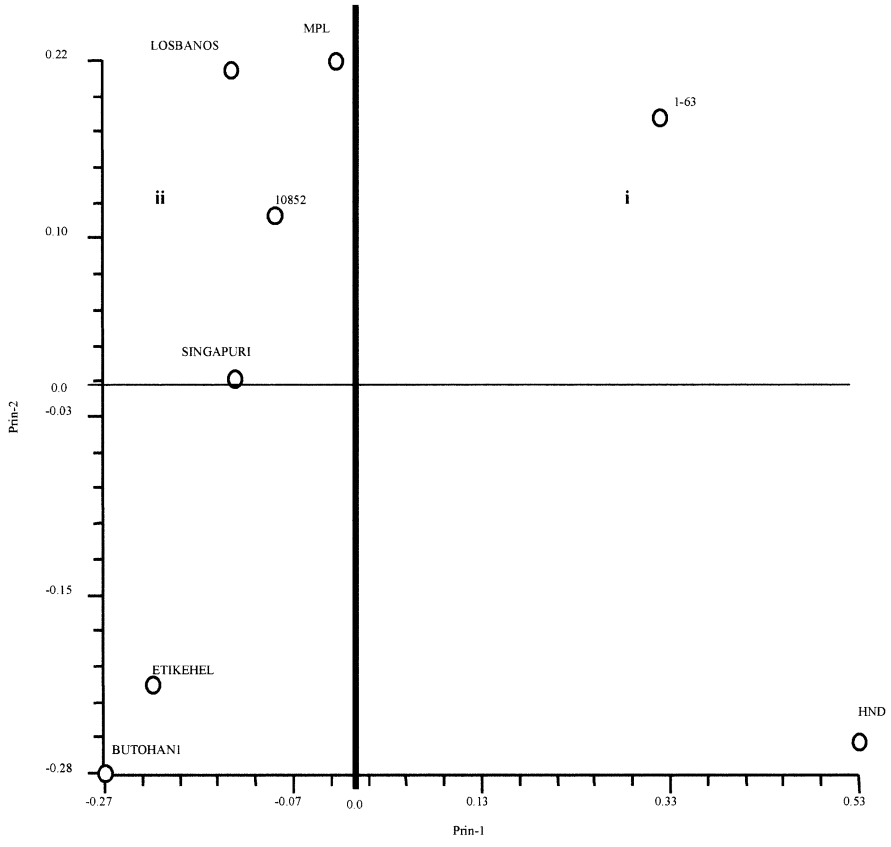

Fig. 4 Principal coordinate map of the *M. balbisiana* accessions. The *thick black line* divides them into two groups: *i* = 1-63 and HND; *ii* = Singapuri, Los Banos, 10852, MPL, Etikehel and Butohan 1

type' accessions were well-separated from 'Pisang lilin' and 'Madang', which clustered together. Subgroup C comprised 'Long Tavoy' and 'Calcutta 4', and was the most-isolated cluster. Neighbor-joining (Fig. 1) and PCO analyses (Fig. 4) portrayed two distinct genetic clusters among the *M. balbisiana* accessions. One group was composed of 'I-63' and 'HND', while the second group consisted of 'Los Banos', 'MPL' (Montpellier), '10852', 'Singapuri', 'Etikehel' and 'Butohan 1'.

Discussion

Relationships within wild diploid *M. acuminata* accessions

The AFLP technique was useful in distinguishing the wild diploid accessions in this study and suggested new subspecies relationships in the *M. acuminata* complex. Our results suggest that there are three subgroups within the *M. acuminata* accessions (Fig. 3). Each subgroup was composed of accessions from different subspecies suggesting that the previous subspecies classification of *M. acuminata*, based entirely on morphological characteristics, isozymes and flavonoids, may not show the genetic relationships among them. Three subspecies were represented in each of subgroups A and B, but ssp. *microcarpa* was the dominant subspecies of A with three accessions, and ssp. *malaccensis* was the dominant subspecies of B with two accessions. Subgroup C was made of two accessions from the spp. *burmannica*. On this basis, it would appear that there are three genetic subspecies in the *M. acuminata* complex.

Perhaps, cross hybridization and chromosome rearrangement among the three subspecies (ssp. *burmannica*, ssp. *malaccensis* and ssp. *microcarpa*) were responsible for the wide genetic variability in the *M. acuminata* complex. Differentiation into subspecies was probably enhanced by geographic isolation. A similar idea for the evolution of the *M. acuminata* subspecies was proposed by Horry and Jay (1988), who suggested the existence of a primitive *M. acuminata* stock from which the other subspecies were derived.

The grouping of the 'Malaccensis holotype' accession with 'Pisang lilin' was not unexpected since the latter is a member of spp. *malaccensis*. However the close affinity of 'Madang' (a ssp. *banksii* derivative) and 'Zebrina' in this group is unusual (Fig. 3). Our study showed that 'SF247', the other ssp. *banksii* representative, grouped with the predominantly ssp. *microcarpa* group A. This confirms that there is wide genetic variability within the ssp. *banksii*, to the extent that some researchers (Cheesman 1948; Argent 1976) suggested raising the subspecies to the category of species (*Musa banksii)*. However, the classification of *M. acuminata* Colla subspecies *banksii* (F. Muell) proposed by Simmonds (1962) is still currently used.

The clustering of 'Selangor' ssp. *malaccensis* in the subgroup dominated by ssp. *microcarpa* was also unex-

pected. Selangor was more closely related to 'Borneo' than the 'Malaccenis holotype'. The ssp. *malaccensis* is widespread geographically, growing at altitudes ranging from sea level to about 500 m and is morphologically very variable (Hari 1989). It is known that Selangor, a geographical region in Asia is dominated by the presence of *M. acuminata* ssp. *malaccensis* genotypes (Shepherd 1988). However, our result indicates that other subspecies may also be present in this area and confusion in classifying the subspecies could have arisen by basing the presence/absence of a subspecies on geographical distribution.

The closeness of 'Truncata' and 'Borneo' may suggest that ssp. *truncata* may also have been derived from the ssp. *microcarpa*. However, 'Borneo' and 'Truncata' were clearly distinguished by three-dimensional PCO analysis (data not shown), supporting their classification as distinct subspecies. In addition, 'Truncata' is characterized with three translocations and is morphologically different from other members of ssp. *microcarpa*, justifying its subspecific status (Shepherd 1988). It is interesting to note that ssp. *zebrina* ('Zebrina') was closer to ssp. *malaccensis*. This suggests that ssp. *zebrina* was derived from the ssp. *malaccensis* (Fig. 3).

The close genetic affinity between 'Long Tavoy' (ssp. *burmannica*) and 'Calcutta 4' (ssp. *burmannicoides*) does not support previous classifications that separated the accessions into two different subspecies (De Langhe and Devreux 1960; Tezenas du Montcel 1988). Our results agree with those of Carreel et al. (1994) and Shepherd (1988), who also reported a close relationship between ssp. *burmannica* ('Long Tavoy') and ssp. *burmannicoides* ('Calcutta 4'), and recognized them as one subspecies (*burmannica*). Shepherd (1988) found more similarities between samples of ssp. *burmannica* and *burmannicoides* from India. Morphological observation of the two subspecies at the field germplasm collection of the International Institute of Tropical Agriculture High Rainfall Station in southern Nigeria, showed that they can be differentiated on the basis of bunch characteristics.

Relationships among *M. balbisiana* accessions

The AFLP data revealed a high level of genetic variability within *M. balbisiana*. This is in agreement with previous reports on the occurrence of wide genetic and morphological variability within the species (Shepherd 1988; Hari 1989; Sotto and Rabara 2000). Subspecies categories have not been reported in *M. balbisiana*, but our data clearly divided the *M. balbisiana* accessions into two groups (Figs. 1 and 4). Whether this may indicate the presence of subspecies requires further work. Nonetheless, the presence of distinct genetic forms in *M. balbisiana* implies that selection of parents may be important for breeding specific traits in any *Musa* improvement program where *M. balbisiana* accessions are used.

Relationships among *M. acuminata* cultivars and the wild diploid accessions

Although *M. acuminata* and *M. balbisiana* are accepted as the progenitors of modern bananas and plantains, the exact subspecies of *M. acuminata* involved in the process are unknown. Similarly, the progenitors of most of the cultivated *M. acuminata* diploids are still largely unknown. Previous studies suggested that the parthenocarpic 'Pisang lilin' was derived from the wild diploids of the ssp. *malaccensis* (Shepherd 1988). While our study partially supports this claim, we also found that 'Pisang lilin' was related to 'Zebrina' (ssp. *zebrina*). Similarly, it appeared that 'Tjau Lagada' was closely related to 'Truncata' (ssp. *truncata*) that is thought to be derived from ssp. *microcarpa*. This study shows that 'SF247', a cultivated ssp. *banksii* accession, may have a hybrid origin since it was distinct from the other ssp. *banksii* accession ('Madang') and clustered with the ssp. *microcarpa* subgroup.

Lebot et al. (1993) stated that triploid AAA cultivars from Papua New Guinea were derived from the contribution of more than one *M. acuminata* subspecies. This was partially supported by the close relationships found in this study between the AAA dessert bananas ('Gros Michel' and Yangambi 'Km 5') and the diploids 'Tjau lagada', 'SF247' and 'Truncata'. This study also showed that 'Gros Michel' and 'Yangambi Km 5' were closely related to subspecies *truncata*, *microcarpa* and *banksii* (Fig. 3), indicating that the three subspecies may have been involved in the origin of the dessert bananas.

In this study, the cooking bananas (ABB) and plantains (AAB) formed two distinct groups that corresponded with the composition of their genomes. The plantains showed very little genetic variability. Unlike the AAA cultivated bananas, the AAB and ABB accessions did not show close relationships with the diploid accessions. Thus, it was not possible to speculate on the origin of these types of bananas from our data. However, it was interesting to note that the ABB group was more closely related to the BB group than was the AAB group.

In conclusion this study showed that AFLP was very useful in: (1) assessing genetic diversity and relationships among accessions of *M. acuminata* and *M. balbisiana*, and (2) identifying relationships between the infra-specific categories of *M. acuminata* and the cultivated edible bananas.

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