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Molecular phylogeny of genus *Musa* determined by simple sequence repeat markers

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Abstract

Musa L. was previously separated into five sections (*Eumusa*, *Rhodochlamys*, *Callimusa*, *Australimusa* and *Ingentimusa*) based on basic chromosome numbers and morphological characters. However, several molecular analyses currently support restructuring of *Musa* species into two sections, *Musa* and *Callimusa*. The application of simple sequence repeat molecular marker analysis to *Musa* phylogeny provided valuable, supplemental information about the classification of, and relationships between, *Musa* species and subspecies. Totally, 28 accessions of *Musa acuminata* Colla subspecies and varieties and 25 accessions of other *Musa* species were evaluated; 12 primers produced 91 polymorphic bands, polymorphic information content ranged from 0.4473 to 0.8394 (average = 0.7226), indicating that the primers showed a high level of polymorphism. Our results generally agreed with previous phylogenetic analyses based on molecular data. One clade comprised species of sections *Australimusa* and *Callimusa* ($X = 10/9$); most species of sections *Eumusa* and *Rhodochlamys* ($X = 11$) formed the other clade. The relationships between most species were as expected; however, some species did not conform to findings of previous studies. A wide range of variability was observed in the *M. acuminata* complex. *M. acuminata* var. *chinensis* and *M. acuminata* subsp. 522 showed the most distant relationships to other subspecies: *Musa laterita*, *Musa ornata* and *Musa velutina* clustered with *M. acuminata* var. *chinensis*, suggesting that they may constitute a secondary gene pool for the improvement of cultivated bananas. Molecular data indicated that *Musa tongbiguanensis* Chen You & Yao-Ting Wu, which was observed and described by our research group in Yunnan, China, was a distinct, new species.

Keywords: banana; evolution; *Musa acuminata* complex; simple sequence repeats; taxonomy

Introduction

Systematic classification of *Musa* began in 1778 when Linnaeus established the *Musa* genus. In 1887, Sagot (1887) suggested that this genus is classified into different sections, namely giant bananas, fleshy and edible bananas, and ornamental bananas. Baker adopted Sagot's suggestion and formally divided *Musa* into three subgenera: *Physocaulis*, *Eumusa* and *Rhodochlamys* (Baker, 1893). Cheesman further divided *Musa* into four sections based on morphology and number of chromosomes: *Eumusa* ($2n = 22$), *Rhodochlamys* ($2n = 22$), *Callimusa* ($2n = 20$) and *Australimusa* ($2n = 20$) (Cheesman, 1947). A new section, *Incertae sedis*, was

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added by Simmonds (1960), and was proposed to include two species: *Musa ingens* Simmonds ($2n = 14$) and *Musa beccarii* Simmonds ($2n = 18$). Simmonds also established the morphological taxonomy of wild *Musa* germplasm (Simmonds, 1960). Argent (1976) placed *M. ingens* into the new section *Ingentimusa*. Häkkinen classified *M. beccarii* into section *Callimusa* (Häkkinen *et al.*, 2005). With the support of several molecular analyses, Häkkinen (2013) restructured *Musa* species into two sections: *Musa* and *Callimusa*. To date, approximately 70 *Musa* species have been reported (Häkkinen, 2013).

Classification of bananas by morphology is limited, and molecular markers are increasingly used in classifying banana germplasm. Previous reports have used molecular markers such as restriction length fragment polymorphisms (RFLP) (Gawel *et al.*, 1992), random amplified polymorphic DNA (Howell *et al.*, 1997), amplified fragment length polymorphisms (AFLP) (Ude *et al.*, 2002; Wong *et al.*, 2002) and PCR-RFLP (Nwakanma *et al.*, 2003) to evaluate the genetic diversity of *Musa*. DNA sequencing techniques have also been widely used in phylogenetic studies of this genus. These techniques include nuclear ribosomal internal transcribed spacers and chloroplast DNA fragments such as *trnL-F* (Li *et al.*, 2010; Liu *et al.*, 2010), *atpB-rbcL*, *rps16* (Li *et al.*, 2010) and *trnT-trnF* (Bekele and Shigeta, 2011).

Simple sequence repeat (SSR) markers pinpoint polymorphisms in numbers of repeats for stretches of consecutively repeated small units (one to six nucleotides) (Condit and Hubbell, 1991). The mutation rate (approximately 10^{-3}) is higher than that for point mutations (approximately 10^{-9}). Consequently, SSRs can generate many more polymorphic markers compared with other methods and can detect recent polymorphisms between closely related accessions (Perrier *et al.*, 2009). SSRs are spread across the genome and are easy to generate; because of their high levels of polymorphism, co-dominance, efficiency and cost-effectiveness, they are widely used in genetic studies. SSR length polymorphism analysis can detect high levels of polymorphism between individuals of *Musa* breeding populations (Crouch *et al.*, 1998, 2000).

Here, SSRs were used to analyse the phylogeny of wild *Musa* species. A total of 53 wild *Musa* species, including 25 species and 28 *Musa acuminata* subsp. were analysed. This intensive sampling and analysis can improve the understanding of the phylogeny of the *M. acuminata* complex and the genus. Our specific objectives were to (1) provide new molecular evidence for the classification of wild *Musa* germplasm resources and (2) to provide valuable information for germplasm collections for cultivated bananas.

Materials and methods

Plant materials and genomic DNA extraction

We analysed accessions of 53 wild species consisting of 25 *Musa* species and 28 *M. acuminata* subsp. Of these accessions, 40 were obtained from Bioversity International's ITC (Supplementary Table S1, available online) and the others were collected from China (Table 1). Identification of the wild *Musa* specimens was performed according to the descriptions of morphological characters provided by Bioversity International (INIBAP/CIRAD, 1996). Genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide protocol (Paterson *et al.*, 1993). Total genomic DNA samples were diluted to 20 ng/μl with sterile H_2O.

SSR analysis

In total, 12 pairs of SSR primers (Supplementary Table S2, available online), synthesized by Invitrogen, Shanghai, were used to analyse the genetic diversity of the *Musa* samples. SSR assays were carried out in a 20 μl reaction

Table 1. Plant materials of the genus *Musa* collected from China

No.	Accession name	Section	Species	Subspecies	Collection location
41	*Musa aurantiaca*	*Eumusa*	*aurantiaca*		21°50.168′N, 101°00.275′E
42	*Musa acuminata*	*Eumusa*	*acuminata*	var. *chinensis*	22°39.170′N, 103°03.763′E
43	*Musa chunii*	*Eumusa*	*chunii*		24°37.049′N, 97°34.954′E
44	*Musa itinerans*	*Eumusa*	*itinerans*		24°38.973′N, 97°35.290′E
45	*Musa tongbiguanensis*	*Eumusa*	*tongbiguanensis*		24°36.955′N, 97°35.154′E
46	*Musa balbisiana*	*Eumusa*	*balbisiana*		24°38.973′N, 97°36.210′E
47	*Musa sanguinea* ssp.1	*Eumusa*	*sanguinea*	Ssp. 1	22°20.558′N, 101°03.361′E
48	*Musa sanguinea* ssp.2	*Eumusa*	*sanguinea*	Ssp. 2	24°02.193′N, 97°38.787′E
49	*Musa yunnanensis*	*Eumusa*	*yunnanensis*		24°43.553′N, 97°34.343′E
50	*Musa nagensium*	*Eumusa*	*nagensium*		24°38.230′N, 97°34.906′E
51	*Musa basjoo*	*Eumusa*	*basjoo*		30°04.775′N, 103°00.250′E
52	*Musa paracoccinea*	*Callimusa*	*paracoccinea*		22°37.111′N, 103°03.764′E
53	*Musa coccinea*	*Callimusa*	*coccinea*		21°50.170′N, 101°00.245′E

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mixture containing 0.125 mM of each deoxy-ribonucleoside triphosphate (dNTP), 10 mM Tris–HCl (pH 8.8), 2 mM $MgCl_2$, 50 mM KCl, 0.08% NP-40, 0.5 μM of each primer, 40 ng genomic DNA and 0.5 U Taq DNA polymerase (Shanghai Sangon, China). Amplification was performed using a Biometra T1 thermocycler (Whatman Biometra, Göttingen, Germany). The PCR reactions were performed as follows: initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 s; annealing at 53–62°C for 45 s and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The amplified products were separated on 8% polyacrylamide gels in Tris-base/boric acid/Ethylene Diamine Tetraacetie Acid (EDTA) buffer. The gels were stained with silver nitrate as described by Zhang *et al.* (2000). PCR analyses were repeated at least twice to ensure repeatability.

Data analysis

The SSR gel images were analysed with BandScan Software version 5.0 (Glyko Inc., Novato, CA, USA; http://www.glyko.com) and confirmed manually. SSR bands were sized and binary coded with 1 or 0 for their presence or absence in each locus/allele, excluding smeared or weak bands. Polymorphism information content (PIC) was calculated for each primer as follows:

$$\mathrm{PIC} = 1 - \sum_{i=1}^{m} p_i^2 - \sum_{i=1}^{m=1} \sum_{j=i+1}^{m} 2p_i^2 p_j^2,$$

where p is the relative frequency of the jth pattern of SSR marker i (Botstein *et al.*, 1980). Genetic similarity coefficients were determined using the index presented in Nei and Li (1979), and the unweighted pair-group method using arithmetic mean (UPGMA) cluster analysis was performed using the Numerical Taxonomy System version 2.1 (NTSYS-pc) program (Applied Biostatistics Inc., New York, NY, USA; Rohlf, 2000). The clustering was also tested by bootstrap analysis using the WinBoot program (Yap and Nelson, 1996, International Rice Research Institute, Manila, Philippines) with 1000 iterations.

Results

Polymorphism of SSR primers

The 12 primer pairs generated polymorphic bands with good repeatability; 91 polymorphic bands were detected. The number of bands generated from each primer ranged from 4 to 15 (average = 7.5). The EST-SSR34 and AGMI67/68 primers generated the largest numbers of bands (11), whereas MA19 produced the fewest bands (4). The PIC was highest in primer EST-SSR34 and lowest in MA19 (average = 0.7226). All primer pairs detected high levels of polymorphism among the samples (Supplementary Table S2, available online).

Phylogenetic analysis of Musa

UPGMA clustering assigned the 53 accessions to two significantly different clusters based on a similarity coefficient of 0.63 (Fig. 1). The first cluster included the accessions of sect. *Musa* and *Musa paracoccinea*, which belong to sect. *Callimusa* with basic chromosome number $X = 10$. The first cluster splits into four branches near a genetic similarity of 0.78. Branch I comprised 18 subspecies of *M. acuminata*. Branch II included eight *M. acuminata* subsp. and four species of sect. *Musa* that have erect inflorescences and are distributed in China. Branch III consisted of 13 species of sect. *Musa* (*Musa itinerans*, *Musa balbisiana*, *Musa nagensium*, *Musa basjoo*, *Musa schizocarpa* subsp. 502, *Musa schizocarpa* subsp. 507, *Musa balbisiana* × *Musa textilis*, *Musa yunnanensis*, *Musa ornata*, *Musa acuminata* var. *chinensis*, *Musa laterita*, *Musa velutina* and *Musa tongbiguanensis*) and one species of sect. *Callimusa* (*M. paracoccinea*). *Musa boman* ($X = 9$) was separated as a single cluster in branch IV.

The second cluster included seven species of sect. *Callimusa*, and 'Marges Elargies' (*M. acuminata* subsp. 522) formed a single branch on the dendrogram with a similarity of 0.74.

Phylogenetic analysis of Musa acuminata *complex*

The UPGMA cluster analysis of 28 accessions of *M. acuminata* complex constructed with SSR markers separated the accessions into six clusters based on a similarity coefficient of 0.77 (Fig. 2): (1) 'Tavoy' (subsp. *burmannica*), 'Calcutta 4' (subsp. *burmannicoides*), 'Khae (Phrae)' (subsp. *siamea*), 'Type 3 ×,' 'Type 2 ×,' 'Pahang IRFA,' 'Pa (Musore) no. 3,' 'Pisang Cici Alas'; (2) 'Malaccensis' (subsp. *malaccensis*), 'Borneo' (subsp. *microcarpa*), 'Truncata' (subsp. *truncata*), 'Hybrid 513,' 'Selangor 2,' 'Higa,' 'Hybrid 507,' 'Pa (Songkhla)'; (3) 'Zebrina' (subsp. *zebrina*), 'Pisang Cici'; (4) 'Banksii' (subsp. *banksii*), 'A3617/9,' 'Hybrid 511,' 'Agutay,' 'Rung Hoa Xoan,' 'THA018,' 'Vietnam no. 5,' 'Makyughu II'; (5) 'Xiao Guo Ye Jiao' (var. *chinensis*); and (6) 'Marges Elargies.'

Discussion

Infrageneric phylogeny and classification of Musa

The genus *Musa* was previously separated into five sections (*Eumusa*, *Rhodochlamys*, *Callimusa*, *Australimusa* and

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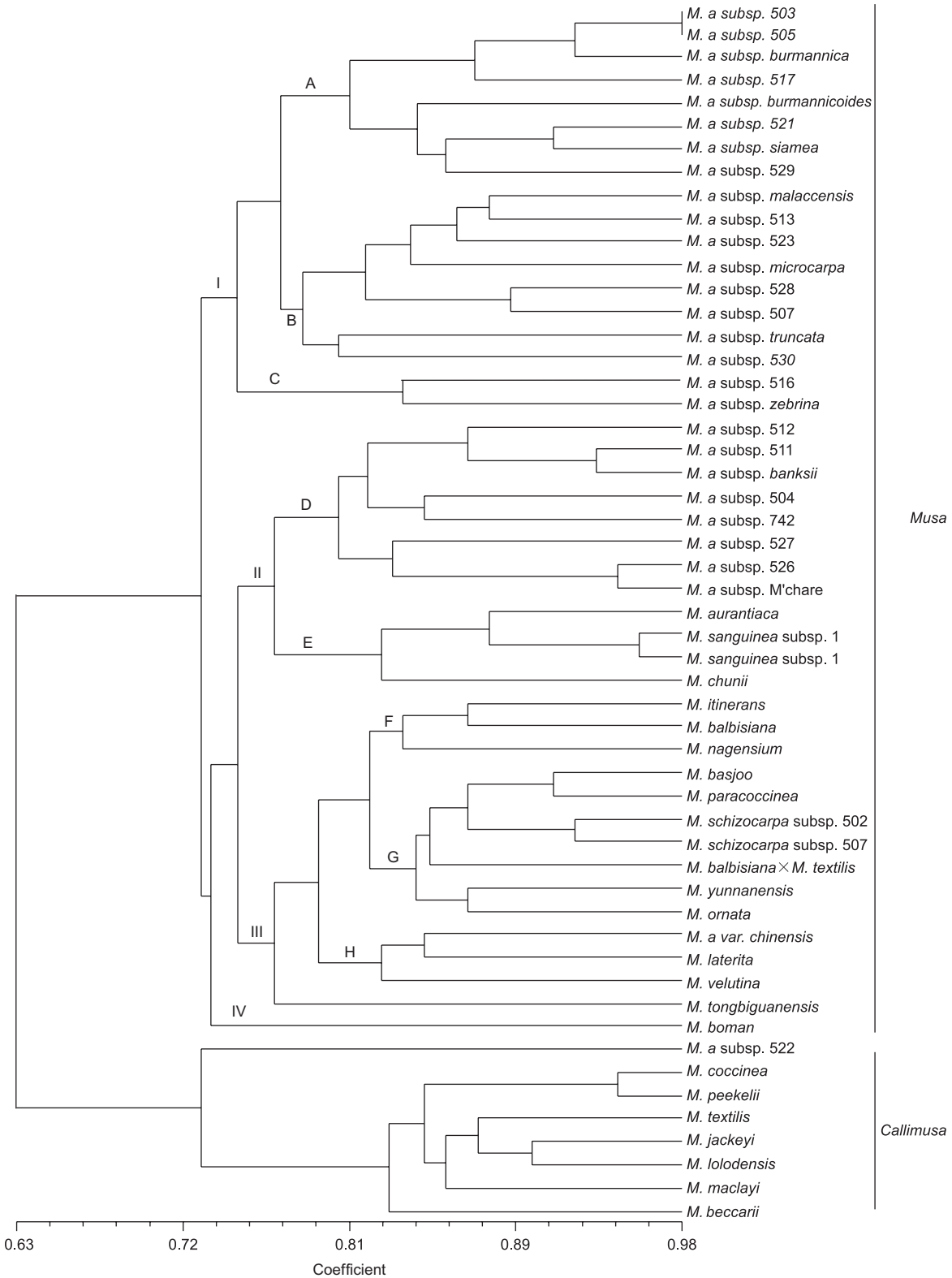

Fig. 1. Dendrogram of genus *Musa* generated with 12 SSR markers using the unweighted pair-group method with arithmetic mean. *M. a, M. acuminata*.

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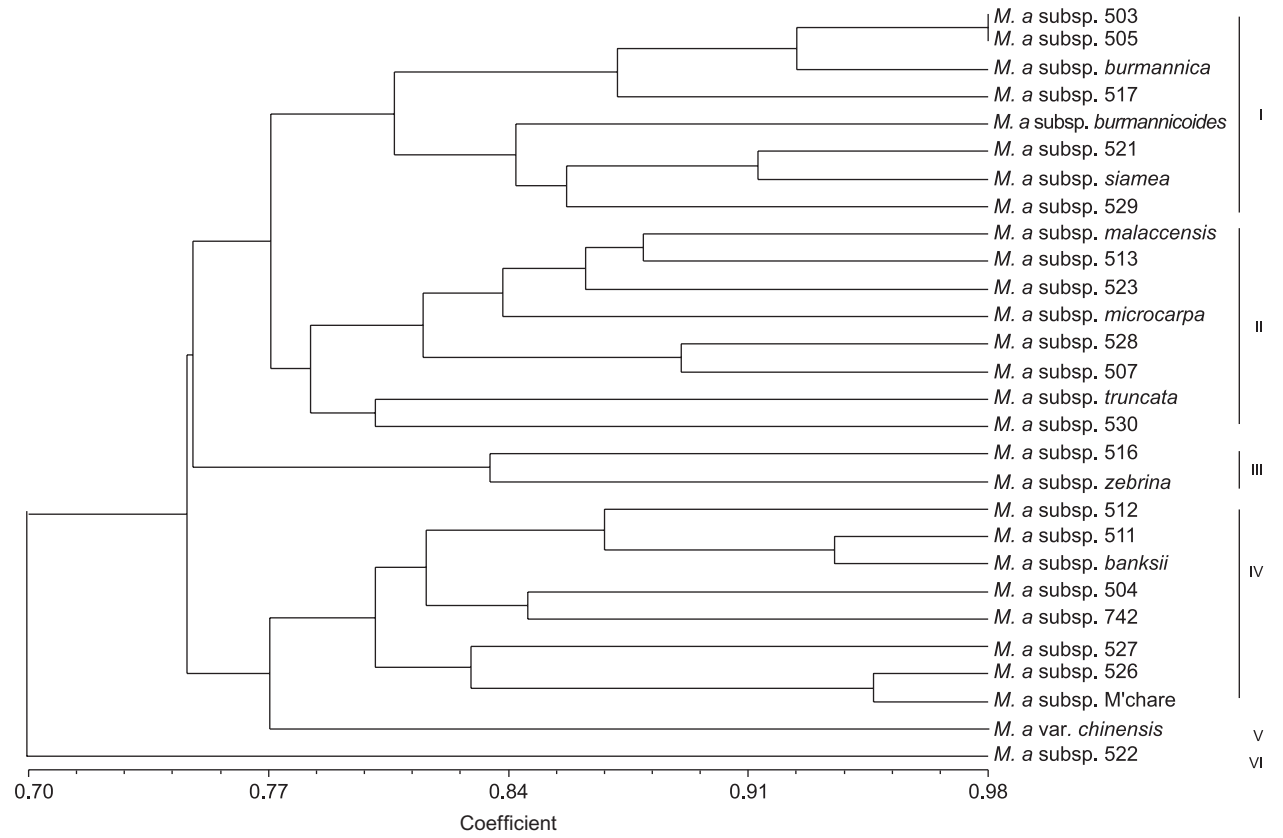

Fig. 2. Dendrogram of *Musa acuminata* (*M. a*) complex generated with 12 SSR markers using the unweighted pair-group method with arithmetic mean.

Ingentimusa) based on basic chromosome numbers and morphological characters (Cheesman, 1947; Simmonds, 1960, 1962; Argent, 1976; Simmonds and Weatherup, 1990). With support from several molecular analyses, Häkkinen (2013) restructured *Musa* species into two sections, *Musa* and *Callimusa*. Our results are largely congruent with those of previous molecular studies (Gawel *et al.*, 1992; Wong *et al.*, 2002; Nwakanma *et al.*, 2003; Liu *et al.*, 2010; Li *et al.*, 2010; Bekele and Shigeta, 2011; Christelová *et al.*, 2011). By SSR-marker UPGMA cluster analysis, 53 accessions were grouped into two significantly different clusters. One cluster comprised species of sect. *Musa* with basic chromosome number of $X = 11$; most species of sect. *Callimusa* ($X = 10/9$) formed the other cluster. These results provide new molecular evidence for sectional relationships in the genus *Musa*.

Relationships within sect. Musa

Although the basic chromosome number of both *Musa* and *Rhodochlamys* was $X = 11$; these sections were separated by differences in morphological characteristics and edibility of fruit. The inflorescences of *Musa* are pendent or semi-pendent; the bracts are commonly green, brown, or dull violet; and the fruit are usually edible. In contrast, inflorescences of *Rhodochlamys* are erect, the bracts are always bright in colour (often red), and the fruit are usually inedible (Cheesman, 1947). Despite these differences, however, there is no significant evidence to support separate classification. SSR analysis revealed cross-clustering of *Musa* and *Rhodochlamys* species. This result was consistent with that of Ude *et al.* (2002); however, Ude proposed that *Rhodochlamys* be considered a separate category. In contrast, we agree with the suggestions of Wong *et al.* (2002) and Christelová *et al.* (2011) that sections *Musa* and *Rhodochlamys* be considered as one category.

Musa chunii, a recently described species (Häkkinen and Hong, 2007), has a close relationship with *Musa aurantiaca* and *Musa sanguinea* (subclade E); each of these species has erect inflorescences and brightly coloured bracts and is distributed in Yunnan, China. *M. laterita* and *M. velutina*, both from sect. *Musa* and having erect inflorescences and brightly coloured bracts, cluster with *M. acuminata* var. *chinensis* (subclade H). Ude *et al.* (2002) maintained that *M. laterita* was closely related to *M. acuminata* and had the closest relationship with *M. acuminata* subsp. *burmannica*. In this study, we examined numerous *M. acuminata* subspecies, including the new variety of

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M. acuminata (*M. acuminata* var. *chinensis*) discovered by Häkkinen and Hong (2007) in China. We observed that *M. laterita* had the closest genetic relationship to *M. acuminata* var. *chinensis* (similarity coefficient = 0.84); the similarity coefficient for *M. laterita* and *M. acuminata* subsp. *burmannica* was only 0.73. *M. ornata*, another species previously of sect. *Rhodochlamys*, has a close relationship with *M. schizocarpa* (subclade G), which is considered to have the S genome for cultivated bananas (Ude *et al.*, 2002). This affinity among *M. laterita*, *M. velutina*, *M. ornata*, *M. acuminata* and *M. schizocarpa* suggests that they may constitute a secondary gene pool for the improvement of cultivated bananas.

M. balbisiana, *M. itinerans* and *M. nagensium*, which form subclade F, are closely related. *M. nagensium* is unique in seed shape and inflorescence structure and has limited distribution in southern Yunnan and northern Myanmar (Liu *et al.*, 2002). *M. itinerans* is unique for having long rhizomes and commonly grows in southern China. Although *M. yunnanensis* and *M. itinerans* have similar-shaped seeds and partly overlap in their distribution ranges (Häkkinen *et al.*, 2008), they clustered in different subclades. *M. yunnanensis* clustered with *M. ornata*, *M. basjoo*, *M. paracoccinea*, and *M. schizocarpa* in subclade G. This is largely in agreement with Li *et al.* (2010).

M. tongbiguanensis is a new species of *Musa* that we observed and described in Yunnan, China (Chen *et al.*, 2008). The vernacular name of *M. tongbiguanensis* is 'lubajiao' (green banana); its main characteristics are large fruit and seeds (seeds are approximately two-fold larger than those of *M. acuminata* var. *chinensis*), and it is only distributed in the Tongbiguan Nature Reserve of Yunnan. Our molecular data indicate that *M. tongbiguanensis* is a distinct, new species. *M. boman* formed a single branch; it was placed in sect. *Australimusa* by Argent (1976) and was confirmed by Gawel *et al.* (1992) based on RFLP analysis. However, our results showed that *M. boman* was closer to sect. *Musa* than to sect. *Callimusa*.

Furthermore, *M. acuminata*, *M. balbisiana* and *M. schizocarpa*, which have the A, B and S genome for cultivated bananas, respectively, were placed into different subclades. The *M. acuminata* complex clustered into subclades A, B, C, D and H; *M. balbisiana* clustered into subclade F; and *M. schizocarpa* clustered into subclade G. Li *et al.* (2010) also observed wide hybridization and suggested that all wild species of this clade should be used as genetic resources for banana breeding. Our results are largely in agreement with that suggestion.

Relationships within sect. Callimusa

Cheesman (1947) suggested that *Australimusa* and *Callimusa* were distinct sections because of significant differences in their seeds. However, we found that these two groups were genetically indistinguishable. The basal position of *M. beccarii* could indicate that it is an independent branch because of unique chromosome numbers ($X = 9$). RFLP analysis of chloroplast DNA showed that *M. beccarii* was more similar to the *M. acuminata* complex (Gawel and Jarret, 1991). Based on AFLP data, Wong *et al.* (2002) found that *M. beccarii* has a close relationship with *Australimusa* species. Our results showed that although *M. beccarii* formed an independent branch, it is closely related to *Callimusa* species.

The relatively close genetic relationship between *M. textilis* and *M. balbisiana* is supported by previous analysis of Chloroplast Deoxyribonucleic Acid (cpDNA) (Gawel and Jarret, 1991). Our result showed that the natural hybrid of *M. balbisiana* × *M. textilis* clustered with *M. schizocarpa*; however, *M. textilis* is closely related to other *Callimusa* species, *Musa jackeyi* and *Musa lolodensis*.

A close relationship was also observed between *Musa coccinea* and *Musa peekelii* subsp. *angustigemma* (similarity coefficient = 0.94).

The grouping of *M. paracoccinea* with sect. *Musa* was unexpected and contrary to previous morphological data (Liu *et al.*, 2002) and molecular data (Liu *et al.*, 2010), which showed that *M. paracoccinea* was closely related to *M. coccinea*. However, we found the closest relationship between *M. paracoccinea* and *M. basjoo* (similarity coefficient = 0.87), whereas the similarity between *M. paracoccinea* and *M. coccinea* was only 0.74.

With a few exceptions, our results were in general agreement with previously published data. Some species (e.g. *M. paracoccinea*) were classified differently than expected, suggesting the need for further investigation. The numbers of *Callimusa* species were also limited in our study, and some recently described species were absent from this analysis. Thus, further study of additional species and morphological characters will be undertaken.

Relationships among Musa acuminata *accessions*

The most prominent species of *Musa* are *M. acuminata* and *M. balbisiana*, which are the wild progenitors donating the A and B genomes, respectively, to banana cultivars (Perrier *et al.*, 2011). *M. acuminata* includes abundant genetic diversity. Totally, ten subspecies and varieties have been reported based on morphological characters: *M. acuminata* subsp. *banksii*, *errans*, *malaccensis*, *zebrina*, *truncata*, *halabanensis*, *siamea*, *microcarpa*, *burmannica*, *burmannicoides* and var. *chinensis* (Feng *et al.*, 2009). Our results grouped *M. acuminata* subsp. into the following six clusters: (1) *burmannica*–*burmannicoides*–*siamea*; (2) *malaccensis*–*microcarpa*–*truncata*; (3) *zebrina*; (4) *banksii*;

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(5) var. *chinensis* and (6) 'Marges Elargies.' Our SSR results were close to those of Perrier *et al.* (2011) who reported the geographical distribution of subspecies of *M. acuminata*: (1) *banksii*: New Guinea; (2) *malaccensis*: Malayan Peninsula; (3) *burmannica–burmannicoides–siamea* complex: South China, Thailand, Myanmar, Bangladesh and northeastern India with sporadic populations southward to Sri Lanka (complex is genetically closer to *malaccensis*); (4) *zebrina/zebrina–microcarpa* complex: Java/Sabah; (5) *truncata*: Malaysian Peninsula; (6) *errans*: Philippines.

Our SSR results showed that *M. acuminata* subsp. *malaccensis*, subsp. *microcarpa* and subsp. *truncata* clustered together but were well separated from subsp. *truncata*. This is largely in agreement with Sequence Related Amplified Polymorphism (SRAP) and AFLP analysis by Muhammad *et al.* (2011). By the geographical distribution, subsp. *malaccensis* and subsp. *truncata* overlap and are close to subsp. *microcarpa* (Perrier *et al.*, 2011).

M. acuminata var. *chinensis*, a new variety of *M. acuminata*, was identified by Häkkinen and is distributed only within China (Häkkinen and Hong, 2007). The vernacular name of *M. acuminata* var. *chinensis* is 'Xiao Guo Ye Jiao' (small-fruited wild banana); it showed a distant genetic relationship to other reported *M. acuminata* subsp. complex.

Our SSR data also showed that *M. acuminata* subsp. 522 was genetically distant from other *M. acuminata* subsp. The local name of *M. acuminata* subsp. 522 is 'Marges Elargies'; this subspecies originated from India and was donated to the Bioversity International ITC in 1988 by France. There was no further information about this subspecies in the *Musa* Germplasm Information System database. In future, we will resample *M. acuminata* subsp. 522 from Bioversity International ITC and will grow it out to identify its morphological characteristics and determine its classification.

These findings suggest that it is important to collect and protect *M. acuminata* var. *chinensis* and *M. acuminata* subsp. 522.

Because of the maternal inheritance of the chloroplast genome and the paternal inheritance of the mitochondrial genome in bananas (Lebot, 1999), further in-depth research on the chloroplast and mitochondrial DNA of *Musa* species is necessary to provide useful information for improving cultivated bananas.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S1479262115000222

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