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RESEARCH PAPER

Genetic diversity of wild banana (*Musa balbisiana* Colla) in China as revealed by AFLP markers

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Abstract Wild banana *Musa balbisiana* Colla is one of the progenitors of cultivated bananas and plantains. It is native to Southeast Asia and the western Pacific. South China represents the northern limit of its distribution range. The genetic diversity of *Musa balbisiana* was assessed by the amplified fragment length polymorphism (AFLP) fingerprinting in 15 populations of China. Four primer pairs produced 199 discernible loci. High levels of genetic diversity were detected, with P = 78.5%, H_E = 0.241, and H_{pop} = 0.3684 at population level, and P = 100%, H_T = 0.3362 and H_{sp} = 0.5048 at species level. Significant genetic differentiation among populations was detected based on Hickory's analysis (27.6%), Shannon's diversity index (27.0%) and AMOVA (27.1%). The AFLP results are discussed and compared with data obtained by microsatellites method. The estimates of genetic diversity and differentiation between each pair of populations computed with microsatellites and AFLP markers were not significantly correlated. Conservation strategies for *Musa balbisiana* in China are proposed.

Keywords AFLP · Genetic diversity · Microsatellites · *Musa balbisiana* · Wild banana

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Introduction

With an annual production of about 100 million tons, bananas (*Musa* spp.) are an important crop in the subtropics and tropics. Southeast Asia is the centre of bananas' domestication. Most of edible bananas originated via hybridization between *Musa balbisiana* Colla and *M. acuminata* Colla. *Musa balbisiana* is native to Southeast Asia and the western Pacific. Populations of South China represent the northern most distribution of the wild banana with a range from tropics (18°40′ N) to subtropics (25°30′ N).

The evaluation and conservation of genetic diversity for the progenitors of cultivated plants is imperative to guarantee sustainable development. Wild banana, *M. balbisiana* Colla, provides important genetic resources for banana breeding because it has numerous agriculturally advantageous characters, such as cold- and disease-resistances. Nevertheless, this species has been under considerable threats in China in the past decades due to the destruction of subtropical evergreen broadleaf forests and other human disturbances. Effective conservation of *M. balbisiana* is urgently needed to preserve the remaining populations for sustaining production of banana. In contrast to the well understood *M. acuminata* Colla, very few population genetics studies to date have been carried out on *M. balbisiana*. Recently, an AFLP analysis on 8 accessions of *M. balbisiana* found high levels of genetic diversity within the species (Ude et al. 2002a, b), which correspond to highly diverged morphological characters across geographical regions (Sotto and Rabara 2000). With its north limit in its distribution range and variable environmental conditions, it is necessary to estimate the level of genetic diversity in natural populations of *M. balbisiana* in China.

Various molecular markers, especially different PCR-based molecular markers including AFLP, RAPD, microsatellites, have been frequently used for assessing genetic diversity and phylogenetic relationship in wild banana and cultivation accessions (i.e., Grapin et al. 1998; Loh et al. 2000; Wong et al. 2001; Carreel et al. 2002; Ude et al. 2002a, b; Creste et al. 2003; Nwakanma et al. 2003). Of fingerprinting techniques, amplified fragment length polymorphism (AFLP) and microsatellite are among the most informative. The two PCR-based marker systems differ in principle, and there are different strengths and limitations. The AFLP technique is based on the selective amplification of restriction fragments obtained from the digestion of total genomic DNA. Given their dominant and biallelic nature, AFLP markers have been increasingly applied to various plants, mainly owing to the capabilities of detecting a very high number of polymorphisms in a single assay, good repeatability and reasonable coverage of the genome (Vos et al. 1995; Cervera et al. 1998; Vuylsteke et al. 1999; Shim and Jørgensen 2000). AFLP has been used to detect the genetic diversity of both cultivated accessions and wild progenitors (*M. acuminata* Colla; Wong et al. 2001). Microsatellites (also called short tandem repeats or simple sequence repeats SSRs; Tautz 1989), in turn, combine several features of an ultimate genetic marker, owing to their abundance and uniform dispersal in genomes, hypervariability, codominant nature, accessibility for other research laboratories (Compbell et al. 2003; Gaudeul et al. 2004). Microsatellites have been widely used to detect the genetic diversity of plant species.

In a molecular breeding study on *Musa*, Crouch et al. (1999) reported poor correlation between estimates of genetic similarities derived from different types of markers. They suggested that such data inconsistency stems from differences among molecular techniques that selectively screened complementary, but not overlapping, regions of the genome. Therefore, integration of genetic estimates from different molecular techniques was proposed to provide a clearer picture of *Musa* genetic relationship and generate highly accurate estimates of genetic similarity in germplasm analysis (Crouch et al. 1999; Wong et al. 2001). In order to obtain a better understanding of the population structure in *M. balbisiana* Colla, the simultaneous use of AFLP and microsatellites will be very informative.

In this study, we analysed the genetic diversity and population structuring in wild banana, *M. balbisiana* Colla, from 15 different populations in China based on AFLP fingerprints. The results were compared to the data of a previous study from the same material produced by microsatellites (Ge et al. 2005). We intended to draw recommendations for conservation purposes based on the comparative analysis of genetic diversity with different markers.

Materials and methods

Sampling

Musa balbisiana Colla ($2n = 22$, Horry et al. 1997) has a wide range in China, with its distribution centred in Guangdong Province. In this

study, a total of 218 individuals from 15 populations of *M. balbisiana* were analysed. Populations were collected from all the provinces in China within the species range, one population each from Fujian, Hainan, and Yunnan, 10 populations of Guangdong, and two populations of Guangxi (Fig. 1, Table 1). About 12–15 individuals were analysed per population. Young, healthy leaves were collected and dried in silica gel. DNA was extracted using the modified CTAB method (Murray and Thompson 1980).

AFLP analysis

AFLP analysis was carried out following Vos et al. (1995) with modifications of the labelling of the *Eco*RI-primers. Near infrared (NIR) fluorescence technology was used for imaging labelled DNA bands. The *Eco*RI+3 primers were labelled with IRD 700 and IRD 800 fluorescence dyes (Li-Cor, Lincoln, NE, USA). After digestion with *Eco*Rl and *Mse*I, adaptors were ligated on both ends of genomic fragments and a two-step selective amplification was performed. We chose four selective primer pairs: M-CAG/E-ACA, M-CAG/E-AAC, M-CTA/E-ACG, M-CTC/E-ACA. Amplification was conducted on a PTC 200 Peltier Thermal Cycler (MJ Research). PCR products were mixed with loading buffer and loaded on 7% polyacrylamide gels after heat denaturation. The products were fractionated on the sequencing system (Li-Cor 4300L; Li-Cor Inc., Lincoln, Nebraska, NE, USA) using electrophoresis. The 50–700 size standard (Li-Cor, Lincoln, NE, USA) was run with the samples to estimate the size of fragments. AFLP patterns were visualized and recorded by SAGA^{MX}3.1 software (Li-Cor, Lincoln, NE, USA). The bands were scored as either present (1) or absent (0) across all loci.

Data analysis

The computer program POPGENE 1.31 (Yeh et al. 1999) was used to provide information on the percentage of polymorphic loci (P), Nei's expected heterozygosity from Hardy–Weinberg assumption ($H_E = 1 - \sum p_i^2$) (Nei 1973) and Shannon's diversity ($H_o = -\sum p_i \log_2 p_i$), where p_i is the frequency of a given AFLP fragments. H_o was calculated at two levels: the average diversity within populations (H_{pop}), and the total diversity (H_{sp}). Then the proportion of diversity among populations was estimated as $(H_{sp} - H_{pop})/H_{sp}$. An analysis of molecular variance (AMOVA) was performed using Arlequin 2.000 (Schneider et al. 2000). The hierarchical analysis was conducted at two levels: (1) among populations; and (2) within populations. A simplified estimate of F_{ST} of Wright (1951) was obtained by AMOVA. As an alternative to AMOVA, population structure was also inferred independently by a

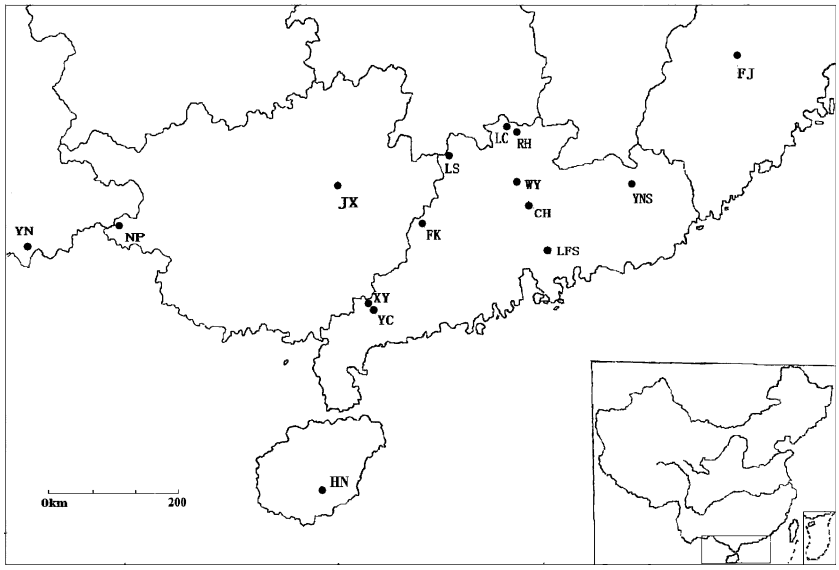

Fig. 1 Sample locations of *Musa balbisiana* Colla in China

Table 1 Population locations of *Musa balbisiana* Colla

Pop. code	Populations	Location	Site coordinate	
1	CH	Conghua, Guangdong	23°45′ N	113°55′ E
2	XY	Xinyi, Guangdong	22°18′ N	111°13′ E
3	LC	Lechang, Guangdong	25°21′ N	113°22′ E
4	FK	Fengkai, Guangdong	23°25′ N	111°53′ E
5	RH	Renhua, Guangdong	25°10′ N	113°45′ E
6	YNS	Yinnashan, Guangdong	24°19′ N	116°02′ E
7	FJ	Sanming, Fujian	26°13′ N	117°36′ E
8	LFS	Luofushan, Guangdong	23°15′ N	113°59′ E
9	YC	Yangchun, Guangdong	22°05′ N	111°22′ E
10	WY	Wongyuan, Guangdong	24°27′ N	113°49′ E
11	LS	Lianshan, Guangdong	24°33′ N	112°01′ E
12	HN	Lingshui, Hainan	18°44′ N	109°52′ E
13	JX	Jinxiu, Guangxi	24°01′ N	110°07′ E
14	NP	Napo, Guangxi	23°27′ N	105°47′ E
15	YN	Malipo, Yunnan	22°59′ N	104°30′ E

Bayesian method using Hickory (0.8) (Holsinger et al. 2002). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram of populations based on AFLP fingerprints was drawn based on pairwise similarities using software TFPGA (version 1.3; Miller 1997).

Results and discussion

Genetic diversity

The four primer combinations resulted in a total of 199 unambiguous bands from 218 plants of 15 populations. At species level, all bands recorded are polymorphic (*P*: 100%), the Nei's heterozygosity (H_T) and Shannon's diversity (H_{sp}) was estimated at 0.3362 and 0.5048, respectively. For the 15 banana populations, the proportion of polymorphic AFLP loci among individuals within populations ranged from 56.8% to 89.9% (Table 2), with an average of 78.5%. The mean of the Nei's heterozygosity (H_E) and the Shannon's diversity (H_{pop}) over the 15 populations is 0.241 and 0.3684, respectively. The highest values of H_E and H_{pop} were detected in Fengkai (FK) of Guangdong and Malipo (YN) of Yunnan, with values of 0.2763 and 0.2764, 0.4210 and 0.4149,

Table 2 Genetic diversity within populations of *Musa balbisiana* Colla

Marker used	AFLP				Microsatellites		
	N	*P*	H_E	H_{pop}	*N*	H_E	*P*
CH	15	65.33	0.2242	0.3334	18	0.2293	40.0
XY	12	56.78	0.2009	0.3001	16	0.4137	80.0
LC	15	83.92	0.2514	0.3872	18	0.2000	40.0
FK	15	89.95	0.2763	0.4210	20	0.3307	80.0
RH	15	70.85	0.2607	0.3845	15	0.2129	60.0
YNS	15	81.41	0.2226	0.3492	20	0.0990	20.0
FJ	14	73.37	0.1375	0.2371	18	0.2178	60.0
LFS	15	88.94	0.2427	0.3823	15	0.5010	80.0
YC	15	87.94	0.2536	0.3929	20	0.3728	80.0
WY	15	87.94	0.2563	0.3987	22	0.2719	100
LS	15	79.90	0.2629	0.3953	14	0.4179	80.0
HN	14	80.90	0.2501	0.3795	16	0.4701	80.0
JX	13	68.84	0.2271	0.3421	20	0.4701	100
NP	15	79.40	0.2720	0.4075	16	0.4836	100
YN	15	82.41	0.2764	0.4149	19	0.6310	100
Mean		78.53	0.241	0.3684		0.3548	80.0
Total	218	100	0.3362	0.5048	267	0.5397	100

N, sample number; *P*, percentage of polymorphic loci; H_E, Nei's expected heterozygosity; H_{pop}, Shannon's diversity

respectively. In contrast, the Sanming (FJ) population of Fujian possessed the lowest level of polymorphism (H_E: 0.1375, and H_{pop}: 0.237) (Table 2).

Compared to other plants (Nybom 2004), both AFLP fingerprinting and microsatellites (Ge et al. 2005) exhibit a high level of intrapopulational genetic diversity in *M. balbisiana* Colla, a result consistent with its morphological and genetic polymorphisms (Sotto and Rabara 2000; Ude et al. 2002a, b). Outcrossing with animal pollination, somatic mutation, and a long life span account for such a high genetic diversity in *M. balbisiana*. In *Musa*, high levels of AFLP polymorphisms were detected in different cultivated accessions and wild banana (Wong et al. 2001; Ude et al. 2002a, b). As parental origins, the genetic materials of wild progenitors are the source for crop's improvement. *Musa balbisiana*, especially that from China, represents an important gene pool for some advantageous features, e.g., cold resistance and drought tolerance. For example, among polyploid cultivars with genomes AB, AAB, and ABB, Dajiao of the ABB type, which is one of the most popular banana landraces in Guangdong Province of China, is much more cold tolerant than cultivars with AAA genome, indicating the contribution of the wild banana to the genetic heterogeneity. With no doubt, the maintenance of biodiversity is an essential prerequisite for the sustainable development and exploitation of hitherto novel crops (Hayward and Sackville Hamilton 1997). Nevertheless, due to the destruction of evergreen broadleaf forests in recent decades, populations of the wild banana has been anthropogenically reduced and fragmented. It is urgent to conserve *M. balbisiana*, especially the northern most populations in China, such as Sanming of Fujian (FJ), Lechang (LC) and Renhua (RH) of Guangdong. Since the Malipo (YN) of Yunnan was revealed with the highest diversity both by AFLP and by microsatellites markers consistently, we recommend this population should also be included for in situ conservation.

Genetic structure between wild banana populations

Analysis of molecular variance (AMOVA; Table 3) revealed that 27.1% of the variation was allocated among populations and 72.9% was due to individual differences within populations. The AMOVA results were corroborated by a Bayesian analysis of population structure. Results of the Hickory analysis gave θ^B value of 0.276, indicating that, on average, 72.4% of AFLP diversity was distributed within the *M. balbisiana* Colla populations and 27.6% between populations. Similarly, the Shannon index calculated from the H_{pop} and H_{sp} relations, allocated 27.0% of the total variation among populations rather than within populations. A UPGMA tree (Fig. 2) based on the pairwise similarities of populations identified two clusters [(CH, XY) and other 13 populations]. These populations were not clustered together according to the geographical distances. Furthermore, the hypothesis of ''isolation by distance'' model was not supported either (R = 0.071; Mantel test).

Both AFLP and microsatellites, which revealed 37% differences between populations (R_{ST} = 0.373; Ge et al. 2005), demonstrated a moderate genetic differentiation. Genetic differentiation among populations is principally a function of gene flow among populations via

Table 3 Analysis of molecular variance (AMOVA) for 218 *Musa balbisiana* Colla individuals among and within populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value*
Among populations	14	1672.89	6.94	27.1	< 0.001
Within populations	203	3789.95	18.67	72.9	< 0.001
Total	217	5462.84	25.61		

*Significance tests (1023 permutations)

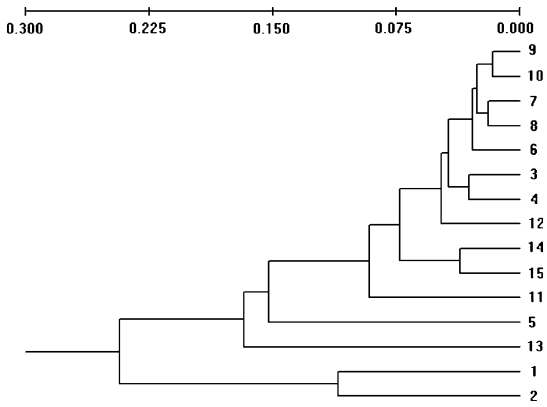

Fig. 2 UPGMA dendrogram of populations of *Musa balbisiana* Colla in China based on AFLP data. Numbers refer to the populations given in Table 1

pollen and seeds dispersal (Loveless and Hamrick 1984). In this study, all of measures used (AMOVA, Hickory's estimate and the Shannon index) resulted in a similar estimate for population differentiation (ca. 27%), which was comparable to the average value of G_{ST} for all categories of plants with outcrossing (0.27) and ingested seed dispersal (0.27) (Nybom 2004). Pollination of *Musa* is mainly by honeybees and bats (Start and Marshall 1976). In China, due to human overexploitation of broadleaf forests, long-tongued fruit bats have decreased dramatically, only honeybees act as the active pollinators for wild banana (Liu et al. 2004). The short flight ranges of the insects plus the limited distance of seed dispersal by gravity or rodents contribute to the restricted gene flow and increase the probability that individuals in close physical proximity mated with one another. Both effects are likely responsible for this present-day structure of genetic variation.

The ultimate aim of this study was to derive conservation decisions based on a comparative survey of genetic diversity conducted with different markers. Populations near the boundary are likely to contain genes not present in the centre of the species range. These genes are likely to be particularly valuable in breeding to broaden the range of a crop, particularly for improving tolerance to stress such as cold, drought, etc. (Hayward and Sackville Hamilton 1997). The moderate genetic differentiation in *M. balbisiana* Colla indicates that sampling for ex situ conservation must cover as many populations as possible. And samples from different habitats, especially those from the north most distribution range, should be considered in conservation.

Comparisons with microsatellites

Since genotyping of *M. balbisiana* Colla has been conducted with various fingerprinting techniques, all of which revealed comparable genotype numbers such as 218 for AFLP and 267 for microsatellites, straight comparisons are therefore possible with the results of our present study. Mantel tests were used to quantify the differences between AFLP and microsatellites results. Although high levels of genetic diversity were revealed by both markers in *M. balbisiana*, the ranking of each population's expected hetrozygosity varies. Except for Malipo (YN) of Yunnan, which was revealed with the highest diversity by both markers consistently, the estimates of genetic diversity for each population varied strikingly between markers. In addition, Microsatellites loci show higher levels of heterozygosity than the AFLP loci (H_E: 0.3548 vs. 0.241) (Table 2). This finding is, nevertheless, consistent with other studies (Mariette et al. 2002). As one of typical dominant markers, AFLPs can only produce two alleles in each locus, and therefore a maximum expected heterozygosity is 0.5, whereas multi-allelic markers like microsatellites can produce values up to one. Furthermore, microsatellites fingerprints are known to exhibit much higher levels of mutation than other parts of the genome (Jarne and Lagoda 1996). In contrast, high-copy genomic regions with lower mutation compared to microsatellites are likely to be targeted at the AFLP fingerprints (Maguire et al. 2002). Poor correlation between estimates of genetic similarity based on AFLP and SSR suggests that both markers may selectively screen complementary, rather than overlapping, regions of the *Musa* genome (Crouch et al. 1999). On the other hand, the relatively few markers being sampled in the AFLP analysis in this study (only 4 primer pairs) may greatly increase the chances of sampling error providing different estimates of genetic diversity.

The genetic differentiation of *M. balbisiana* Colla in China revealed by microsatellites is

about 37% (R_{ST} = 0.373; Ge et al. 2005). Both AFLP and microsatellites demonstrated that a high proportion of the genetic variation was partitioned within population. However, estimates of differentiation between each pair of populations computed with microsatellites and AFLP markers were not significantly correlated (R = 0.1187; Mantel test). In *Musa* breeding study, it has been shown that the different molecular markers may provide different pictures (Crouch et al. 1999). Thus, it is necessary to utilize a range of marker systems in order to generate highly accurate estimates of genetic similarity in genetic diversity study. *Musa balbisiana* is widely distributed from Papua New Guinea, Indonesia, Malaysia, Philippine to Thailand and India, This work only includes the samples from China and this result may not represent the whole genetic variation across its natural range. In order to overcome this limitation, future genetic analysis should include samples from its whole range.

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