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ORIGINAL PAPER

Identification of cytoplasmic ancestor gene-pools of *Musa acuminata* Colla and *Musa balbisiana* Colla and their hybrids by chloroplast and mitochondrial haplotyping

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Abstract Cytoplasmically inherited characters such as resistance to viral and fungal diseases, determination of starch types, crop yield, resistance to low or high temperature often contribute to the advantageous phenotypic traits of plants. In the present study, our goal was to elucidate the genealogy of cytoplasmic genomes chloroplast and mitochondria in banana. Banana breeding is rather complicated because of the low fertility and mostly unknown origin of the edible cultivars, therefore, knowledge on the putative fertile ancestors of cytoplasmic genomes chloroplast and mitochondria would be beneficial for breeding programmes. Based on the established marker systems distinct species specific gene-pools could be identified for both chloroplast and mitochondrial genomes for *Musa acuminata* and *Musa balbisiana* wild types, respectively. Detailed analysis of the species specific chloroplast and mitochondrial gene-pools of *M. acuminata* and *M. balbisiana* revealed six chloroplast and seven mitochondrial gene-pools in the analysed accessions. Comparative analysis of the haplotypes revealed the presence of Primary Centers of origin for both chloroplast and mitochondrial genomes of both species supporting the idea of common origin of these genomes. Cytotypes representing combinations of *M. acuminata* chloroplast and mitochondrial gene-pools were identified in majority of the analysed hybrid cultivars. A single *M. acuminata* cytotype was present in the majority of the analysed cultivars, which combination was not detected in any of the wild types. On the other part a single *balbisiana* cytotype was identified participating in the formation of interspecies hybrids. The strong preference for the presence of certain cytoplasmic gene-pools in cultivars may indicate hundreds of years of natural as well as of farmers' selection supplementing the phenotypic traits provided by the nuclear genome. Based on the present results the present day subspecies classification of *M. acuminata* is also discussed.

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Introduction

Musa (Musaceae) is one of the most important staple crops widely cultivated in tropics and subtropics. The present day edible bananas originate mostly from the diploid species ($2n = 22$) *Musa acuminata* and *M. balbisiana* (Cheesman 1950; Simmonds 1995). The diploid or polyploid cultivated banana varieties are mostly sterile intra- or inter-specific hybrids of these two species and have been fixed through hundreds of years of human selection. The haploid genome originating from *M. acuminata* is called the 'A' genome while that of *M. balbisiana* is the 'B' genome. Based on morphological characters and ploidy level five main genetic groups (AA, AB, AAA, AAB, ABB) have been described for cultivated bananas by Simmonds and Shepherd (1955) and Simmonds (1962). Several subspecies have also been recognised on morphological characters and hybridisation studies in *M. acuminata* wild types: *banksii*, *burmannica*, *burmannicoides*, *errans*, *malaccensis*, *microcarpa*, *siamea*, *truncata* and *zebrina*, however their classification was questioned latter based on molecular data (Carreel et al. 2002; Ude et al. 2002). Despite the observed high genetic diversity in *M. balbisiana* (Sotto and Rabara 2000) less effort was devoted to the classification of this species.

Banana breeding using high quality cultivars must follow complicated regimes (Ortiz 2003) since most of the edible cultivars are low fertility triploids mostly with unknown origin. However, knowledge on the putative fertile ancestors would be beneficial for breeding programmes. Several attempts were made for the elucidation of the origin of banana cultivars using various molecular techniques (see for example Ude et al. 2002; Nair et al. 2005). Recently, Swangpol et al. (2007) used comparative sequencing of the chloroplast genome for lineage assessment of interspecific hybrids. Recently, Raboin et al. (2005) could identify putative wild type ancestors of Gros Michel triploid AAA cultivar using nuclear genome based molecular tools.

Cytoplasmic genomes—chloroplast and mitochondria—provide photosynthetic carbon fixation and oxidative phosphorylation, respectively, for the plant cell. Beyond these basic functions, however, there were many traits reported being cytoplasmically inherited in plants such as resistance to viral and fungal diseases (Voluevich and Buloichik 1992; Braun et al. 1989), determination of starch types (Chen and Zhu 1999), crop yield (Loessl et al. 2000), resistance to low or high temperature (Hutton and Loy 1992; Shonnard and Gepts 1994), control of tissue culture regeneration ability (Ekiz and Konzak 1991) and influence on programmed cell death (Balk and Leaver 2001). Therefore, elucidating the genealogy of the cytoplasmic genomes of cultivars and wild types would be beneficial for banana breeding. In *Musa* the chloroplast genome was shown to be maternally, while the mitochondrial genome paternally inherited (Faure et al. 1993). In previous studies using cytoplasmic genome based marker systems the paternal and maternal lineages of the different *Musa* genotypes could be traced (Carreel et al. 2002), the sectional relationships of *Musa* could be revealed (Nwakanma et al. 2003) and geographical differences in the gene-pool of the chloroplast cytotypes could be identified in *M. balbisiana* populations (Ge et al. 2005).

The objective of this study was to reveal the genealogy of the cytoplasmic genomes of diploid wild as well as the present day intra- and inter-specific *Musa* accessions thus identifying the putative ancestor gene-pools also contributing to the hybrid genotypes. Therefore, we analysed the relationship of the chloroplast and mitochondrial haplotypes of wild type versus diploid, triploid and tetraploid cultivars in order to elucidate their genealogy.

Materials and methods

Sample set

Purified DNA of 48 members of a 'mini core' collection of 51 accessions (http://www.musagenomics.org/index.php?id=137) isolated by Isabelle Hippolyte (CIRAD) was kindly provided by the Global Musa Genomics Consortium. Additionally, three *M. balbisiana* (samples 50, 51 and 52), two *M. acuminata* (samples 9 and 10) and one *M. schizocarpa* (sample 54) wild types kindly provided by J. Dolezel (Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany Olomouc, Czech Republic) were also used for the present study. The DNA of the latter samples was purified using Qiagen Plant mini kit. Altogether ten *M. acuminata*, eight *M. balbisiana*, one *M. schizocarpa* wild types and 35 diploid, triploid and tetraploid cultivars were analysed during the course of the present study (Table 1).

PCR-RFLP of the organellar genomes

The procedure for analysing genetic diversity of cytoplasmic genomes, the method originally described by Dumolin-Lapègue et al. (1997) was used with minor modifications (see below). This way eight chloroplast and four mitochondrial primer pairs were used to amplify selected regions of the organellar genomes (Table 2). PCR amplification of these chloroplast and mitochondrial regions were performed in 25 µl reaction mixture containing 0.2 µM of each primer, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.2 unit HotStarTaq polymerase (QIAGEN), 1× PCR buffer supplied with the polymerase and 25 ng genomic DNA. Amplifications were run in PTC-100 (MJ Research Inc.) PCR machines using the following cycling conditions: activation of the polymerase was accomplished by incubating the reaction mix at 94°C for 15 min, followed by 35 cycles of 94°C 45 s, T_{an} (Table 2) 45 s and 72°C for D_{el} (Table 2) followed by 72°C for 10 min elongation in the last cycle. Subsequently, the fragments were restriction digested with the enzymes listed in Table 2. The banding patterns were visualised either by polyacrylamide gel electrophoresis (PAGE) followed by silver staining (PCR-RFLP 1-11; Table 2) or by Agarose gel electrophoresis using 1% Agarose (Sigma) in TBE and subsequently staining the gels with 0.3 µg/ml Ethidiumbromid (PCR-RFLP 12; Table 2). The PAGE analysis was made in 0.5 mm thick 8% gels (Rotiphorese Gel 30, Roth) prepared in 1× TBE using GelBond®PAG Film (Cambrex, USA) gel support film. The gels were loaded using shark tooth combs, which allow the detection of small differences in the mobility of the fragments. Dual Vertical Slab Gel System DSG-250 (CBS) electrophoresis system cooled to 15°C was used for running the gels at 500 V for 3 h. Subsequently, the support film attached gels were fixed in 10% ethanol and 0.5% acetic acid for 30 min and then stained in 0.1% $AgNO_3$ for 45 min. The bands were developed by soaking the stained gels in solution containing 1.5% NaOH, 0.01% $NaBH_4$ and 0.15% formaldehyde until bands appeared. Developing was terminated by soaking the gels in 0.75% Na_2CO_3 for 5 min.

Table 1 List of analysed accessions and their haplotype affiliations

Number	Nuclear genotype	Species	Sub-species	Common name	Country of origin	INIBAP transit centre code	Chloroplast haplotype	Mitochondrial haplotype	Chloroplast gene-pool	Mitochondrial gene-pool	Cytotype
1	AA	*acuminata*	*microcarpa*	Borneo	Malaysia, S/E Borneo	ITC0253	18	4	Ca3	Ma2	IV
2	AA	*acuminata*	*burmannicoides*	Calcutta 4	India, Calcutta	ITC0249	11	4	Ca1	Ma2	I
3	AA	*acuminata*	*errans*	Agutay	Philippines	ITC1028	6	2	Ca2	Ma1	III
4	AA	*acuminata*	*siamea*	Khae (Phrae)	Thailand	ITC0660	34	4	Ca1	Ma2	I
5	AA	*acuminata*	*burmannica*	Long Tavoy		ITC0283	12	4	Ca1	Ma2	I
6	AA	*acuminata*	*banksii*	Paliama	Papua New Guinea (PNG067)	ITC0766	21	2	Ca2	Ma1	III
7	AA	*acuminata*	*banksii*	Banksii	Papua New Guinea	ITC0623	20	3	Ca2	Ma1	III
8	AA	*acuminata*	*zebrina*	Zebrina	Indonesia	ITC1177	9	7	Ca1	Ma3	II
9	AA	*acuminata*	*zebrina*	Maia Oa	Hawaii	ITC0728	10	7	Ca1	Ma3	II
10	AA	*acuminata*	*malaccensis*	Malaccensis	Peninsular Malaysia	ITC0250	13	4	Ca1	Ma2	I
11	AA	AAcv (18)	Pisang jari buaya	Pisang Jari Buaya	Malaysia, kelatan, Thai border	ITC0312	8	8	Ca1	Ma4	XI
12	AA	AAcv (2)	Sucrier	Pisang mas	Malaysia	ITC0653	1	2	Ca1	Ma1	VIII
13	AA	AAcv	Cooking AA	Tomolo	Papua New Guinea (PNG023)	ITC1187	16	5	Ca1	Ma1	VIII
14	AAA	AAA	Cavendish	Grande Naine	Guadeloupe	ITC0180	15	2	Ca1	Ma1	VIII
15	AAA	AAA	Cavendish	Petite Naine		ITC0654	14	2	Ca1	Ma1	VIII
16	AAA	AAA	Cavendish	Poyo	Nigeria	ITC0345	14	2	Ca1	Ma1	VIII
17	AAA	AAA	Orotava	Pisang Kayu,	Indonesia (IDN098)	ITC0420	7	2	Ca1	Ma1	VIII
18	AAA	AAA	Ambon	Pisang bakar,	Indonesia (IDN106)	ITC1064	7	2	Ca1	Ma1	VIII
19	AAA	AAA	Gros Michel	Gros Michel	Guadeloupe	ITC0484	14	2	Ca1	Ma1	VIII
20	AAA	AAA	Rio	Leite		ITC0277	19	2	Ca3	Ma1	X
21	AAA	AAA	Lujugira/Mutika	Mbwazirume	Burundi	ITC0084	23	6	Ca2	Ma3	IX
22	AAA	AAA	Lujugira/Mutika	Intokatoke	Burundi	ITC0082	24	6	Ca2	Ma3	IX
23	AAA	AAA	Ibota	Yangambi km5	DR Congo	ITC1123	17	5	Ca1	Ma1	VIII
24	AAB	AAB	Nadan	Lady Finger	India	ITC0582	2	2	Ca1	Ma1	VIII
25	AAB	AAB	Pome/Prata	Foconah	DR Congo	ITC0649	2	2	Ca1	Ma1	VIII
26	AAB	AAB	Pome/Prata	Prata Ana	Brazil	ITC0962	2	2	Ca1	Ma1	VIII
27	AAB	AAB	Plantain	Orishele	Nigeria	ITC1325	22	2	Ca2	Ma1	III
28	AAB	AAB	Plantain	Red Yade	Cameroon	ITC1140	21	2	Ca2	Ma1	III
29	AAB	AAB	Silk	Figue Pomme Géante	Guadeloupe	ITC0769	3	1	Ca1	Ma1	VIII
30	AAB	AAB	Popoulou/ Maia Maoli	Popoulou	Cameroon	ITC0335	21	2	Ca2	Ma1	III

Table 1 continued

Number	Nuclear genotype	Species	Sub-species	Common name	Country of origin	INIBAP transit centre code	Chloroplast haplotype	Mitochondrial haplotype	Chloroplast gene-pool	Mitochondrial gene-pool	Cytotype
31	AAB	AAB	Pisang raja	Pisang Raja Bulu	Indonesia (IDN093)	ITC0843	27	2	Cb1	Ma1	XII
32	AAB	AAB	Nendra padaththi	Pisang Rajah	Malaysia	ITC0243	3	2	Ca1	Ma1	VIII
33	AAB	AAB	Mysore	Pisang Ceylan	Malaysia	ITC1441	5	2	Ca1	Ma1	VIII
34	AB	ABcv		Safet Velchi	India	ITC0245	4	9	Ca1	Ma1	VIII
35	AB	ABcv		Kunnan	India, Kerala	ITC1034	3	1	Ca1	Ma1	VIII
36	ABB	ABB	Klue teparod	Kluai Tiparot	Thailand (THA020)	ITC0652	28	10	Cb1	Mb1	V
37	ABB	ABB	Pelipita	Pelipita	Philippines	ITC0472	22	10	Ca2	Mb1	XIV
38	ABB	ABB	Bluggoe	Dole		ITC0767	3	10	Ca1	Mb1	XIII
39	ABB	ABB	Saba	Saba	Philippines	ITC1138	4	10	Ca1	Mb1	XIII
40	ABB	ABB	Monthan	Monthan	India	ITC0046	4	10	Ca1	Mb1	XIII
41	ABB	ABB	Peyan	Simili Radjah	From India through DR Congo	ITC0123	27	10	Cb1	Mb1	V
42	ABB	ABB	Ney mannan	Ice Cream		ITC0020	4	10	Ca1	Mb1	V
43	ABB	ABB	Pisang Awak	Namwa Khom	Thailand (THA011)	ITC0659	26	10	Cb1	Mb1	V
44	ABBT	ABBT	ABBT *Ssp*/sgr 501	Yawa 2	Papua New Guinea (PNG072)	ITC1238	27	11	Cb1	Mb1	V
45	BB	*balbisiana*	type 4	Pisang Klutuk Wulung	Indonesia (IDN056)	ITC1063	30	10	Cb1	Mb1	V
46	BB	*balbisiana*	type 4	Pisang Batu,	Indonesia (IDN080)	ITC1156	27	10	Cb1	Mb1	V
47	BB	*balbisiana*	type 1	Honduras	Honduras (seeds)	ITC0247	36	10	Cb1	Mb1	V
48	BB	*balbisiana*	type 3	Lal Velchi	India	NEU0051	37	10	Cb1	Mb1	V
49	BB	*balbisiana*		Tani		ITC1120	29	12	Cb1	Mb1	V
50	BB	*balbisiana*		Type Cameroon	Sri Lanca	ITC0246	31	13	Cb2	Mb3	VII
51	BB	*balbisiana*		Singapurii		ITC0248	33	14	Cb2	Mb2	VI
52	BB	*balbisiana*		Butuhan	Philippines	ITC0564	32	14	Cb2	Mb2	VI
53	AS	AS		Wompa	Papua New Guinea (PNG063)	ITC1152	25	2	Cs	Ma1	_
54	SS	*schizocarpa*			Papua New Guinea	ITC0890	35	15	Cs	Ms	_

Table 2 List of PCR-RFLPs used for haplotyping the chloroplast and mitochondrial genomes along with the generated fragment size and the corresponding annealing temperature (T_{an}), elongation time (D_{el}) and restriction enzyme (RE) used for generating the PCR-RFLP profiles

PCR-RFLP	Locus discriptor	Size (bp)	T_{an} (°C)	D_{el}(min)	RE	Forward primer	Reverse primer	References
Chloroplast								
1	psaA [PS I (P700 apoprotein A1)]/trnS [tRNA-Ser (GGA)	3,840	62	4	*Hinf*I	5′-ACTTCTGGTTCCGGCGAACGAA-3′	5′-AACCACTCGGCCATCTCTCCTA-3′	Demesure et al. (1995)
2	trnC [tRNA-Cys (GCA)]/trnD [tRNA-Asp (GUC)]	4,220	58	4	*Taq*I	5′-CCAGTTCAAATCTGGGTGTC-3′	5′-GGGATTGTAGTTCAATTGGT-3′	Demesure et al. (1995)
3	trnD [tRNA-Asp (GUC)]/trnT [tRNA-Thr (GGU)]	1,550	55	2	*Taq*I	5′-ACCAATTGAACTACAATCCC-3′	5′-CTACCACTGAGTTAAAAGGG-3′	Demesure et al. (1995)
4	tRNAleu-tRNAleu (tRNAleu intron 1)	500	58	1	*Cfo*I	5′-CGAAATCGGTAGACGCTACG-3′	5′-GGGGATAGAGGGACTTCAAC-3’	Taberlet et al. (1991)
5	MatK (maturase gene)	3,400	54	4	*Taq*I	5′-AACCCGGAACTAGTCGGATG-3′	5′-CTCAATGGTAGAGTACTCGG-3′	Nishikawa et al. (2002)
6	tRNAlys (trnK gene)	3,200	58	4	*Hinf*I	5′-AACCCGGAACTAGTCGGATG-3′	5′-TCAATGGTAGAGTACTCGGC-3′	Tsumura et al. (1996)
7	trnL-trnF [tRNALeu (UAA)/tRNA-Phe(GAA)]	350	54	0.5	*Cfo*I	5′-TCCGTCGACTTTATAAGTCGTG-3′	5′-TGCCAGGAACCAGATTTGAACT-3′	Nishikawa et al. (2002)
8	trnL-trnF [tRNALeu (UAA)/tRNA-Phe(GAA)]	330	54	0.5	*Hinf*I	5′-GATTTGAACTGGTGACACGAG-3′	5′-AAAATCGTGAGGGTTCAGTC-3′	Jung et al. (2004)
Mitochondria								
9	nad1exonB/nad1exonC	1,400/1,800	57.5	2	*Alu*I	5′-GCATTACGATCTGCAGCTCA-3′	5′-GGAGCTCGATTAGTTTCTGC-3′	Demesure et al. (1995)
10	rps14/cob	1,300	54.5	2	*Alu*I	5′-CACGGGTCGCCCTCGTTCCG-3’	5’-GTGTGGAGGATATAGGTTGT-3′	Demesure et al. (1995)
11	ccb203	420	56	1	*Hinf*I	5′-ASGTTCTACGGACCGATGCC-3′	5′-CACGGGGAGGGAGCRGGCGA-3′	Duminil et al. (2002)
12	*cox* II (cytochrome oxidase subunit II gene intron)	850/1,000/1400	52	2	*Hinf*I	5′-TAGRAACAGCTTCTACGACG-3′	5′-GRGTTTACTATGGTCAGTGC-3′	Duminil et al. (2002)

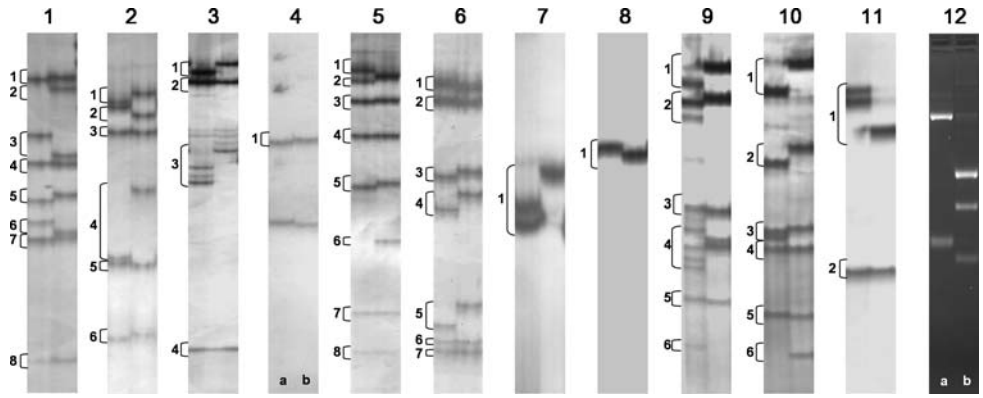

Fig. 1 Examples of eight chloroplast and four mitochondrial PCR-RFLP patterns. Amplified regions are numbered as in Table 2. PCR-RFLPs 1–11 were analysed on polyacrylamide gel with silver staining, while PCR-RFLP 12 was visualised on agarose gels. *Brackets* indicate the bands/regions considered for scoring. Since only two examples are shown not all the scored regions show polymorphism. In PCR-RFLP #4 only the upper band was considered, since the lower one showed symmetrical polymorphisms. PCR-RFLP #9 shows the polymorphisms obtained with the 1,800 bp fragment. PCR-RFLP #12 shows the variation of the 1,000 bp fragment; **a** *M. acuminata*, **b** *M. balbisiana*. PCR-RFLP #4 shows the mutation discriminating Cavendish accessions Grand Naine (**a**) and Petit Naine, Poyo (**b**)

Generating haplotype reads

Ten out of the 12 PCRs gave similar sized amplificates. For describing the banding patterns in these cases the PCR-RFLP data were scored as multistate unordered characters. Each clearly visible and recognizable polymorphic restriction band on the polyacrylamide gel was regarded as a character/locus and its states as different alleles (Fig. 1) since band migration on the acrylamide gel system is also influenced by internal sequence differences of the fragments (data based on *Quercus* chloroplast amplificates not shown). All PCR-RFLP profiles were determined at least in duplicates (independent PCR amplification and subsequent restriction digestion). In Fig. 1 the scored bands are indicated as regions and all minor differences in the mobility of the fragments revealed by using shark tooth combs were recorded as alleles of the particular band. This way up to seven polymorphic bands could be detected in a single PCR-RFLP combination (Table 3), while each band yielded several alleles. On the contrary the mitochondrial markers 9 and 12 gave different PCR fragment sizes. PCR-RFLP 9 resulted in two fragment sizes. *M. acuminata* was characterized by a 1,400 bp while *M. balbisiana* type mitochondria by 1,800 bp long PCR fragments, respectively. No variation was observed in the 1,400 bp long fragment, while the 1,800 bp long fragment yielded different PCR-RFLP patterns (Fig. 1-9). Cox II (PCR-RFLP 12) gave three different fragment sizes. In *M. acuminata* type mitochondria 800, 1,000 and 1,200 bp long amplificates were observed, while exclusively 1,000 bp fragments were detected in *M. balbisiana* type mitochondria. RFLP variation was observed in the 1,000 and 1,200 bp long fragments. Because of the variation of the original fragment length along with additional RFLP variations this marker was scored as pattern (Fig. 1-12). The combination of all these alleles within an individual formed a haplotype. This way a 28 digit string of loci could be established for the chloroplast haplotype and a 14 digit string for the mitochondrial haplotype, respectively.

Table 3 Number of polymorphic markers used for principal component analysis in all the samples and in the species specific gene-pools of *M. acuminata* and *M. balbisiana* type chloroplast (Ca and Cb) and mitochondria (Ma and Mb)

PCR-RFLP chloroplast	No. of bands	Polymorph in all	Polymorph in Ca	Polymorph in Cb
1	8	5	3	0
2	6	4	4	1
3	4	3	2	2
4	1	1	1	0
5	8	6	4	2
6	7	7	6	1
7	1	1	1	1
8	1	1	1	0
Sum	36	28	22	7
PCR-RFLP mitochondria	No. of bands	Polymorph in all	Polymorph in Ma	Polymorph in Mb
9	6	6	0	6
10	6	5	4	2
11	2	2	1	1
128[a]	1	1	1	1
Sum	15	14	6	10

[a] Scored as pattern. See 'Generating haplotype reads' in "Material and methods"

Statistical analysis of the haplotypes

Genetic relationships of the haplotypes were analysed by principal component analysis (PCA) based on correlation using the PAST software package (Hammer et al. 2001). Grouping of haplotypes based on PCA analysis was verified by molecular variance analysis (AMOVA) using the Arlequin v3.1 (Excoffier et al. 2005) software package to estimate variance components (between groups and between haplotypes within groups).

Minimum spanning tree (MST) has been computed for assessing the relatedness of cytoplasmic haplotypes using the Arlequin v3.1 (Excoffier et al. 2005) software package. The trees were visualized in TreeView v1.6.6 (Page 1996) and latter redesigned in MSPowerPoint.

Results

In order to elucidate the resolution power of uniparentally inherited chloroplast and mitochondrial marker systems in identifying the putative ancestor cytoplasmic gene-pools, a collection of 54 accessions was analysed consisting of 52 *Musa* accessions representing different variants of the 'A' and 'B' nuclear genotypes supplemented by one *M. schizocarpa* homozygote and one *M. acuminata* × *M. schizocarpa* hybrid (Table 1). Based on preliminary experiments eight regions representing 28 polymorphic bands/loci of the chloroplast and four regions of the mitochondrial genomes representing 14 polymorphic bands/loci were selected and analysed (Table 2).

Diversity analysis of the chloroplast genome

The number of polymorph bands per primer-enzyme combinations ranged from 1 to 7 in the eight analysed regions of the chloroplast genome (Table 3). Altogether 28 polymorph bands could be identified for haplotyping the chloroplast genome yielding, 37 different chloroplast haplotypes among the 54 analysed. Four haplotypes (two BBw; 47 and 48, one ABcv; 34 and the SS 54) were incomplete, since the PCR amplification of locus 2 and in two samples (34 and 48) also locus 1 was not successful despite of several attempts.

Principal component analysis (PCA) confirmed by AMOVA giving the highest variance among groups (72.66%, $P < 0.00001$) revealed three species specific gene-pools based on full haplotypes (28 markers), since all three gene-pools were characterised by the exclusive presence of either *M. acuminata* (AA) or the *M. balbisiana* (BB) or *M. schizocarpa* (SS) wild type accessions along with diploid and polyploid cultivars (Fig. 2a). Both the *M. acuminata* (Ca) and the *M. balbisiana* (Cb) specific gene-pools were further analysed by PCA excluding the uniform markers present in the haplotypes within groups. PCA based on the residual 22 polymorphic markers (Table 3) present in the Ca haplotypes revealed three sub-groups (Fig. 3a). AMOVA confirmed this genetic structure, yielding the highest variance among groups (48.1%; $P < 0.00001$). The presence of diploid wild types *M. acuminata* ssp. *burmannicoides* (Calcutta 4; 2), *siamea* (4), *burmannica* (5), *malaccensis* (10) and *zebrina* (8 and 9) characterized the largest gene-pool (Ca1) along with the three AA cultivars (11, 12 and 13) and hybrids representing all triploid combinations (AAA: 14, 15, 16, 17, 18, 19, 23; AAB: 24, 25, 26, 29, 33 and ABB: 32, 38, 39, 40 and 42) as well as two diploid AB hybrids (34 and 35). The three Cavendish accessions (14, 15 and 16) were also found in this gene-pool along with the Gros Michel sample (19). *M. acuminata* wild types [ssp. *errans* (3) and *banksii*; 6 and 7] marked the Ca2 gene-pool together with six hybrids (AAA: 27, 28, 30, 37; AAB: 21, 22). *M. acuminata* ssp. *microcarpa* (1) wild type and one hybrid (AAA: 20) formed the third gene-pool (Ca3). On the other part PCA analysis of the Cb haplotypes based on seven polymorphic marker loci (Fig. 3b; Table 3) revealed three subgroups. AMOVA confirmed this structure yielding majority of variance among groups (74.9%; $P < 0.00293$). *M. balbisiana* wild type genotypes (45, 46, 48 and 49) featured the larger gene-pool along with four triploid (AAB: 31; ABB: 36, 41, 43) and the single tetraploid (44) cultivars. Three diploid *M. balbisiana* wild types 50, 51 and 52 formed a second, while 47 a third gene-pool in Cb with no related cultivars in our sample set.

Diversity of the mitochondrial genome

Altogether 14 polymorphic markers could be identified in the four analysed mitochondrial regions for diversity assessment of the mitochondrial haplotypes (Table 3). Based on these markers 15 different mitochondrial haplotypes could be distinguished in the 54 analysed accessions. Similar to chloroplast haplotyping PCA based comparison of the complete haplotypes (14 markers) revealed three species specific gene-pools (Fig. 2b). AMOVA yielded 81% variance among groups ($P < 0.00001$). The larger gene-pool (Ma) included 36 accessions harbouring all the mitochondrial haplotypes found in the *M. acuminata* wild types (samples 1–10). Seventeen accessions formed a smaller gene-pool (Mb) including all the *M. balbisiana* wild types (samples 45–52). The single *M. schizocarpa* wild type formed the third species-specific gene-pool along with the single AS hybrid (53). Additional structuring by PCA of the Ma group based on 6 within group polymorphic markers (Table 3) revealed four gene-pools of the mitochondrial haplotypes (Fig. 4a). Analysis of the molecular variance (AMOVA) of the group structure revealed 74.22% variance

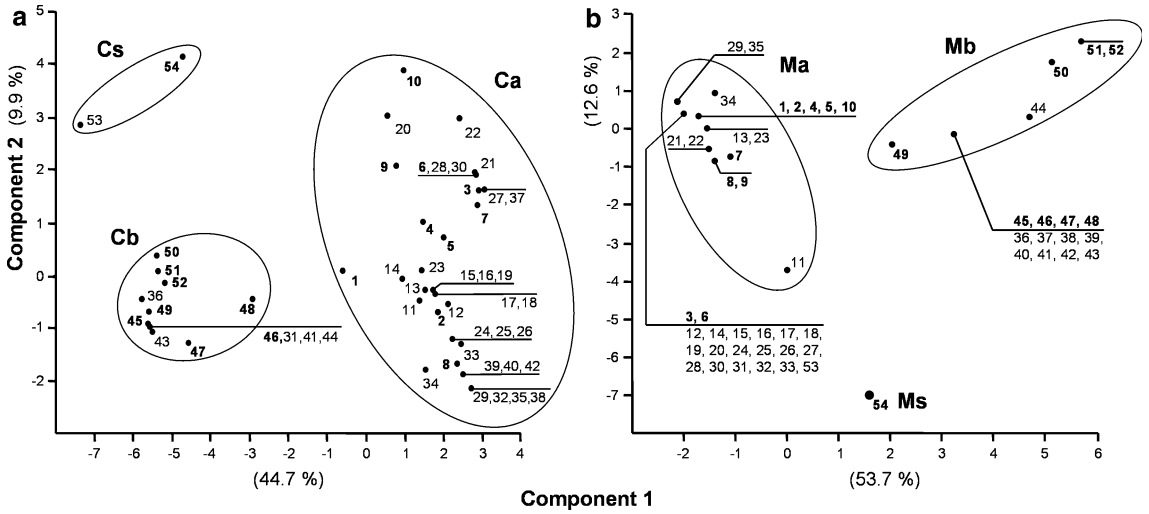

Fig. 2 Discrimination of chloroplast haplotypes (**a**) based on complete 28 loci haplotypes, and mitochondrial haplotypes (**b**) based on full 14 loci haplotype reads by principal component analysis. Wild type haplotypes are indicated in *bold*. Identical haplotypes are collected around a line pointing to the position of the respective samples. Numbering of accessions corresponds to Table 1. Percent contribution of the component to variability is indicated in *parenthesis*

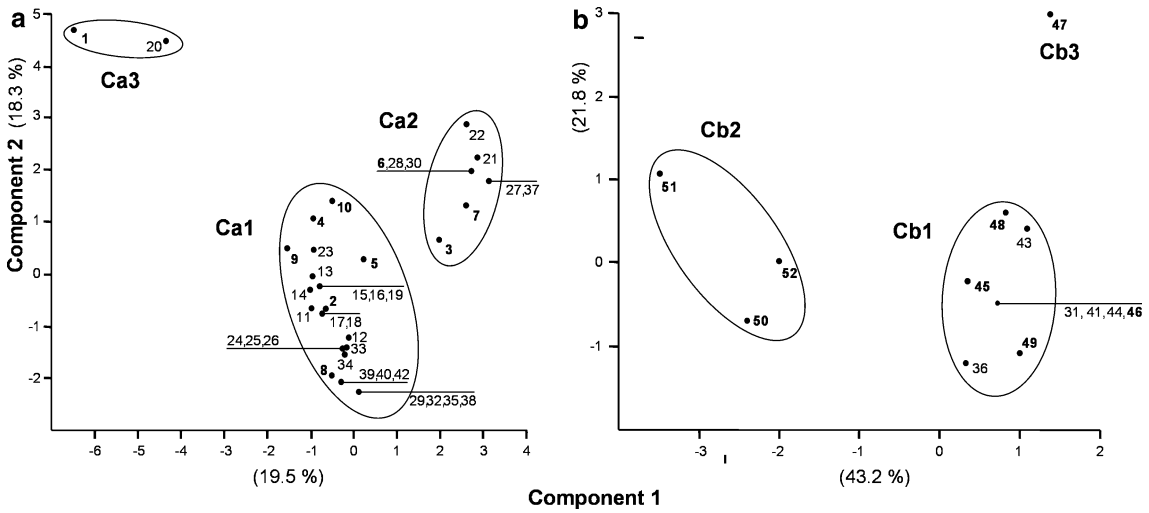

Fig. 3 Detailed analysis of *M. acuminata* and *M. balbisiana* chloroplast gene-pools using restricted haplotype strings by omitting uniform markers. **a** *M. acuminata* type chloroplast gene-pools based on 22 loci haplotypes. **b** *M. balbisiana* type chloroplast gene-pools based on seven loci haplotypes. Wild type haplotypes are indicated in *bold*. Identical haplotypes are collected around a line pointing to the position of the respective samples. Numbering of accessions corresponds to Table 1. Percent contribution of the component to variability is indicated in *parenthesis*

among groups ($P < 0.00001$). The gene-pool (Ma1) was marked by wild types *M. acuminata* ssp. *errans* and *banksii*, while one diploid cultivar (12), two diploid hybrids (34 and 35) and 18 triploid hybrids consisting of seven AAA (14, 15, 16, 17, 18, 19, 20), ten AAB (24, 25, 26, 27, 28, 29, 30, 31, 32, 33) formed the rest of the haplotypes. The AS (*M. acuminata* × *M. schizocarpa*) hybrid cultivar (53) was also found in this gene-pool. *M. acuminata* ssp. *macrocarpa*, *burmannicoides*, *siamea*, *burmannica* and *malaccensis*, one diploid AA (13) and one triploid cultivar (23) formed gene-pool Ma2. The third gene-pool (Ma3) was represented by two identical wild type haplotypes of *M. acuminata* ssp. *zebrina* and two AAA hybrids (21 and 22) again with identical haplotypes. Cultivar 11 (AA) showed a very distinct mitochondrial haplotype suggesting the existence of an additional gene-pool (Ma 4) not represented by wild types in our sample set. Concerning the Mb group (Fig. 4b) PCA analysis based on ten within group polymorphic markers (Table 2) revealed that wild types 45, 46, 47, 48 and 49 form a gene-pool (Mb1) along with nine hybrids, representing all the ABB and the single ABBT accessions. Wild types 51, 52 (Mb2) and 50 (Mb3) indicate the presence of two additional gene-pools without related cultivars present in our collection. AMOVA confirmed this

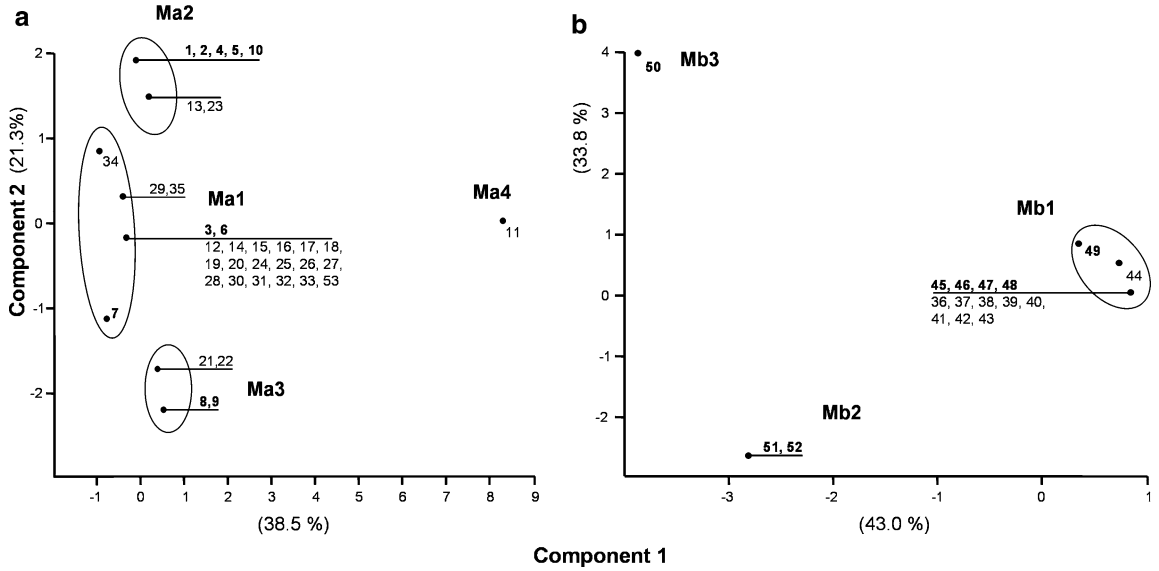

Fig. 4 Detailed analysis of *M. acuminata* and *M. balbisiana* mitochondrial gene-pools using restricted haplotype strings by omitting uniform markers. **a** *M. acuminata* type mitochondrial gene-pools based on six loci haplotypes. **b** *M. balbisiana* type mitochondrial gene-pools based on ten loci haplotypes. Wild type haplotypes are indicated in *bold*. Identical haplotypes are collected around a line pointing to the position of the respective samples. Numbering of accessions corresponds to Table 1. Percent contribution of the component to variability is indicated in parenthesis

genetic structure yielding 69.7% variance among groups ($P < 0.00001$).

Genealogy of the cytoplasmic genomes

Minimum spanning trees based on pair wise distance estimation has been computed for revealing the genealogy of the chloroplast and mitochondrial haplotypes (Fig. 5). The applied marker system was not based on sequence data and evidence obtained on oak chloroplast fragments showed that changes in migration of bands in acrylamide gels was also dependent on their sequence composition additionally to length variation (Burg unpublished). Consequently, the observed alterations in fragment migration may also be the result of several mutations. Therefore, the genetic distances were assessed as arbitrary operational units (AOU). This way *M. acuminata*, *M. balbisiana* and *M. schizocarpa* haplotypes could be well differentiated for both chloroplast and mitochondria. The distance for the closest chloroplast haplotypes was 12 and 13 AOU for *acuminata/balbisiana* and *balbisiana/schizocarpa*, respectively. As far as the mitochondrial haplotypes were concerned *acuminata/balbisiana* relation spanned 10 AOU distance, while for *acuminata/schizocarpa* relation this was only seven. The genetic distances observed between species were equal or higher than those observed within species. The existence of subgroups revealed by PCA could be verified by identifying lineages in MST (Fig. 5a, b). Comparing MST of *acuminata* and *balbisiana* chloroplast haplotypes primary centres of origin (PCO) of closely related haplotypes could be observed in each species (Fig. 5). In *acuminata* this was represented by wild types ssp. *burmannicoides*, ssp. *burmannica* and ssp. *siamea*; chloroplast haplotypes 11, 12 and 34, respectively of the Ca1 gene-pool (Fig. 5a). Within this gene-pool the chloroplast haplotypes of ssp. *zebrina* and ssp. *malaccensis* were somewhat more distantly related (chloroplast haplotypes 9, 10 and 13). The Ca2 gene-pool was represented by the closely related haplotypes of ssp. *banksii* and ssp. *errans* (chloroplast haplotypes 20, 21 and 6), which was a well separated branch of the PCO observed in Ca1. Similarly the chloroplast haplotype of ssp. *microcarpa* (chloroplast haplotype 18) originated from the PCO present in Ca1. Based on the genetic distances between haplotypes smaller genetic diversity was observed in *M. balbisiana* chloroplast haplotypes. However, PCO in the Cb1 gene-pool represented by Pisang Klutuk Wulung, Pisang Batu and Tani (chloroplast haplotypes 30, 27 and 29) could also be identified. The other two gene-pools were branches of the PCO found in Cb1. As far as *M. schizocarpa* haplotypes were concerned they were distantly related to *M. balbisiana*.

Concerning mitochondrial haplotypes in *M. acuminata* the haplotypes were genetically close to each other except mitochondrial haplotype 8. Primary centre representing the Ma1 gene-pool may be identified including the wild type haplotypes 2 and 3. Branches of this primary centre represented the other three gene-pools (Fig. 5b). Longer genetic distances were observed in case of *M. balbisiana* where a PCO represented by the haplotypes 10 and 12 could be recognised (Mb1 gene-pool). Gene-pools Mb2 and Mb3 were branches of this PCO.

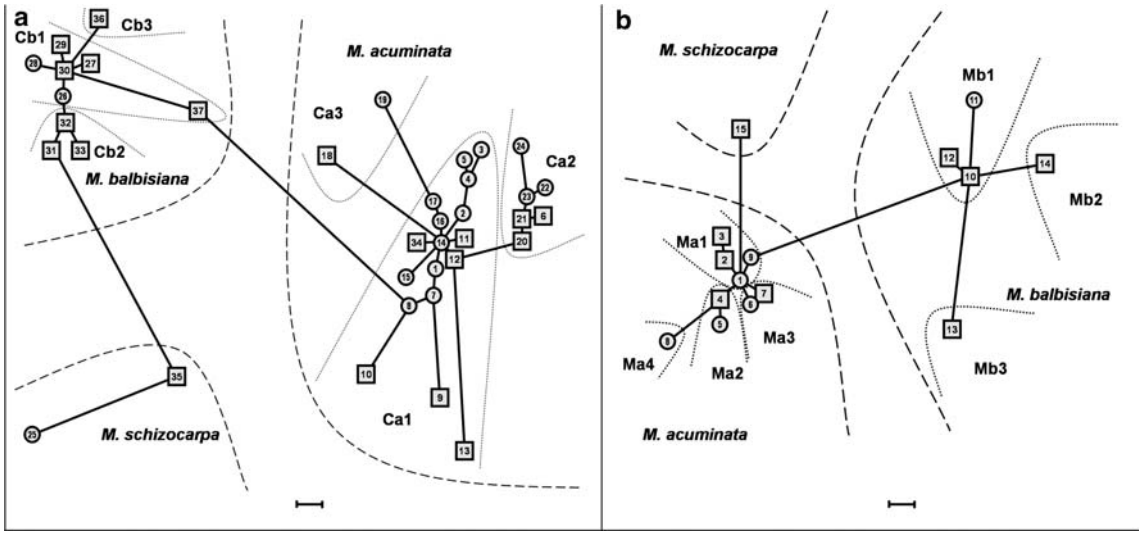

Fig. 5 Minimum spanning tree of chloroplast (**a**) and mitochondrial (**b**) haplotypes. The *bar* represents an arbitrary operational unit (AOU) for estimation of the genetic distances of the haplotypes. *Square symbols* represent haplotypes of wild types. The haplotype numbers for chloroplast and mitochondria correspond to those listed in Table 1

Cytotype diversity of the accessions

Altogether 14 cytotypes (combination of chloroplast and mitochondrial gene-pools) were identified in the analysed samples. The analysed ten wild type *M. acuminata* accessions yielded four cytotypes, while three cytotypes were recorded in the eight *M. balbisiana* wild types (Table 4). These were different from the cytotype observed in *M. schizocarpa*. As far as the 35 cultivars were concerned nine different cytotypes could be identified. Only two out of

Table 4 Cytotypes identified in the sample set and the corresponding wild types representing the respective gene-pool

Nuclear genotype	Wild types representing the chloroplast gene-pool	Cytotype number	Cytotype combination	Number of accessions	Wild types representing the mitochondrial gene-pool
AAA	2, 4, 5, 10;8, 9	VIII	Ca1/Ma1	7	3, 6, 7
	3, 6, 7	IX	Ca2/Ma3	2	8, 9
	1	X	Ca3/Ma1	1	3, 6, 7
AAcv	2, 4, 5, 10; 8, 9	VIII	Ca1/Ma1	2	3, 6, 7
	2, 4, 5, 10; 8, 9	XI	Ca1/Ma4	1	–
AAB	2, 4, 5, 10; 8, 9	VIII	Ca1/Ma1	6	3, 6, 7
	3, 6, 7	III	Ca2/Ma1	3	3, 6, 7
	45, 46, 47, 48, 49	XII	Cb1/Ma1	1	3, 6, 7
ABB	2, 4, 5, 10; 8, 9	XIII	Ca1/Mb1	4	45, 46, 47, 48, 49
	3, 6, 7	XIV	Ca2/Mb1	1	45, 46, 47, 48, 49
	45, 46, 47, 48, 49	V	Cb1/Mb1	3	45, 46, 47, 48, 49
ABcv	2, 4, 5, 10; 8, 9	VIII	Ca1/Ma1	2	3, 6, 7
ABBT	45, 46, 47, 48, 49	V	Cb1/Mb1	1	45, 46, 47, 48, 49
AAwt	2, 4, 5, 10	I	Ca1/Ma2	4	2, 4, 5, 10
	8, 9	II	Ca1/Ma3	2	8, 9
	3, 6, 7	III	Ca2/Ma1	3	3, 6, 7
	1	IV	Ca3/Ma2	1	1
BBwt	45, 46 47, 48, 49	V	Cb1/Mb1	5	45, 46, 47, 48, 49
	51, 52	VI	Cb2/Mb2	2	51, 52
	50	VII	Cb2/Mb3	1	50

the nine cytotypes resembled those found in the wild types (Cytotypes III and V; Table 4), while the rest of the cytotypes represented new combinations.

Intraspecies M. acuminata hybrids

Three different cytotypes (VIII, IX and X) could be identified in the ten triploid AAA genotypes (Table 4). Cytotype VIII was found most frequently. None of these combinations reflected those found in *acuminata* wild types. All three Ca gene-pools were present in these cultivars along with two mitochondrial gene-pools (Table 4). The chloroplast genome of seven triploid hybrids including Cavendish and Gros Michel (14–19 and 23) belonged to the Ca1 gene-pool represented by wild types *M. acuminata* ssp. *burmannicoides*, *siamea*, *zebrina* and *malaccensis*, the mitochondrial genome of these accessions originated from Ma1 gene-pool marked by wild types ssp. *errans* and *banksii* representing Cytotype VIII. A similar mitochondrial type was identified in accession 20 (Rio); however, its chloroplast genome represented the Ca3 gene-pool marked by *M. acuminata* ssp. *microcarpa*. The remaining two AAA hybrids had Ca2 chloroplast and Ma3 mitochondrial genomes representing the gene-pools marked by *M. acuminata* ssp. *errans* and *banksii* for chloroplast and ssp. *zebrina* for mitochondria, respectively (Cytotype IX). Similar to the triploids two out of the three diploid cultivars possessed cytotype VIII (12 and 13), while the third one was a single representative of cytotype XI having Ca1 chloroplast along with a unique *M. acuminata* type mitochondrial genome (Ma4), which was not represented in *M. acuminata* wild types analysed.

Interspecies hybrids

Different cytotypes could be identified in the triploid plantain (AAB) and cooking banana (ABB) cultivars. While mostly *acuminata* type cytoplasms were identified in plantains (III and VIII) a mixed cytotype (XII) was present in Pisang rajah (31). Similar to the intraspecies hybrids cytotype VIII was overrepresented since six (24, 25, 26, 29, 32 and 33) out of the ten analysed accessions were of this cytotype (Table 4). On the contrary cooking banana cultivars contained either mixed (XIII and XIV) or pure balbisiana cytotype (V), this latter was identical to wild types 45, 46, 47, 48 and 49. Similar *M. balbisiana* type V cytoplasm was identified in the tetraploid hybrid as well. On the contrary both AB diploid cultivar possessed Cytotype VIII *acuminata* type cytoplasm.

Discussion

Based on the 52 analysed *M. acuminata*, *M. balbisiana* and their hybrid accessions we could identify 36 chloroplast and 15 mitochondrial haplotypes, which revealed six chloroplast and seven mitochondrial gene-pools representing the descendants of ancient chloroplast and mitochondrial genomes. The analysed wild types represented all gene-pools identified in hybrid cultivars except one mitochondrial gene-pool (Ma4), which was represented by a diploid cultivar.

Genealogy of chloroplast and mitochondrial gene-pools in *M. acuminata* and *M. balbisiana*

Minimum spanning tree analysis revealed PCOs in both chloroplast and mitochondrial genealogy. MST analysis suggested a PCO within the Ca1 chloroplast gene-pool including several closely related haplotypes including the wild types ssp. *siamea*, spp. *burmannicoides* and spp. *burmannica* representing the North-western distribution area of *M. acuminata*. The two more distantly related wild types ssp. *zebrina* and ssp. *malaccensis* possibly represents geographic variants of the Ca1 gene pool; the former representing the eastern part of the chloroplast distribution area (Indonesian islands), while ssp. *malaccensis* a local variant representing peninsular Malaysia. The PCO identified within Ca1 may be considered as origin for the Ca2 as well as for the Ca3 gene pools. The Papua New Guinea distribution area represented by wild types ssp. *banksii* and ssp. *errans* showed already distinct evolution. The biphyletic origin of Ca3 gene-pool may be explained by the few haplotypes identified in this group lacking intermediate haplotypes connecting this gene pool to the PCO.

The PCO of the mitochondrial haplotypes of *M. acuminata* was represented by ssp. *banksii*, ssp. *errans*; the gene-pool represented in most of the hybrid genotypes.

These data indicate that the evolution of present day banana cultivars is based on a restricted number of ancestors as far as the cytoplasmic genomes are concerned.

Origin of hybrids

Based on the identified chloroplast and mitochondrial gene-pools all hybrid combinations could be deduced supposing hybridisation of different wild cytotypes. Exempt was the diploid cultivar Pisang Jari Buaja (AAcv), which's mitochondrial haplotype was representing a unique gene-pool not including any of the analysed wild types.

Twenty out of the analysed 33 hybrid accessions possessed Cytotype VIII (Ca1/Ma1) *acuminata* type cytoplasm. The existence of Cytotype VIII could be explained by crossing of Cytotype I or II maternal and Cytotype III paternal ancestors. This way subspecies *burmannica*, *burmannicoides*, *siamea*, *malaccensis* or *zebrina* may be supposed as maternal, while *banksii* or *errans* as paternal ancestor. The reciprocal cross is represented by Cytotype

IX (Ca2/Ma3) found in the Lujugira/Mutika group suspecting ssp. *banksii* or *errans* as maternal and ssp. *zebrina* as paternal ancestor as was also suggested by Carreel et al. (2002). The triploid AAA cultivar Rio was shown to originate from ssp. *microcarpa* as maternal and ssp. *banksii* or *errans* as paternal ancestors. Previously it was reported that ssp. *banksii* and *errans* are involved in formation of almost all diploid and triploid cultivars and parthenocarpic varieties. (Carreel et al. 2002; Lebot 1999) In our classification both of these subspecies belong to Cytotype III consequently these subspecies could be the paternal ancestor of cultivars providing the Ma1 mitochondrial type or occasionally the Ca2 chloroplast type to the hybrids. Additionally, Cytotype III was identified in three of the plantain cultivars, possibly indicating the participation of ssp. *banksii*, *errans* gene-pool in formation of these hybrids as well. Recently, Swangpol et al. (2007) reported ssp. *malaccensis* as possible donor of the chloroplast genome to the AAA triploid cultivar Grande Naine (Cavendish group), which is in accordance with our findings as well.

The presence of Cytotype VIII in majority of the analysed cultivars may reflect the selective advantage either of this cytoplasmic combination or the combination of the nuclear genomes beyond. The restricted presence of the reciprocal cross represented by Cytotype IX (Ca2/Ma3) may favour the selective advantage of Cytotype VIII for hybrid formation, however to explain this phenomenon needs further studies.

Concerning *M. balbisiana* there was only a single cytotype, which could be identified as participant in interspecies (A × B) hybrid formations (Cytotype V). This cytotype included the accessions Honduras, Lal Velchi, P. Klutuk Wulung, P.Batu and Tani (morphotypes 1, 3, 4, 4 and 4, respectively, as suggested by Horry). This cytotype provided its mitochondrial genome to all cooking banana accessions suggesting as being pollen donor in these hybrids indicating a putative preference for *balbisiana* mitochondria in hybrids of *balbisiana* driven nuclear genomes (ratio of genomes 2–1 for B genome) contrasting plantains having *acuminata* type mitochondria in *acuminata* driven triploid nuclear genomes. The maternal contribution of this cytotype could also be observed in cooking bananas and in a single plantain hybrid as well.

Cytotypes versus subspecies

Ude et al. (2002) has suggested that subspecies classification based on morphological, isoenzyme and flavonoid characteristics may not reflect their genetic relationship. Based on AFLP analysis they suspected three subspecies in the *acuminata* complex, dominated by the subspecies *microcarpa*, *malaccensis* and *burmannica*.

In our study, we could confirm partly this classification since the ten analysed *M. acuminata* wild types representing eight subspecies yielded four cytoplasmic combinations. This way cytotype I subspecies *burmannica*, *burmannicoides*, *siamea*, and *malaccensis* could be supposed representing only varieties of the north-western part of the *acuminata* distribution area (India, Thailand, Malaysia and Philippines) representing the "burmannica" and "malaccensis" groups suggested by Ude et al. (2002). The similarity of ssp. *burmannica* and ssp. *burmannicoides* on cytotype level has already been suggested by Carreel et al. (2002). Additionally, we also found ssp. *microcarpa* (cytotype IV) as representative of a distinct gene-pool confirming Ude's suggestion. However different cytotype ancestor may be supposed for *M. acuminata* ssp. *banksii* and ssp. *errans* (cytotype III) representing the Papua New Guinea region along with the Philippines indicating that the geographic borders listed here are not very strict. We could confirm previous finding of Carreel et al. (2002) that *M. acuminata* ssp. *zebrina* (cytotype II) has a distinct cytotype representing the Indonesian islands. However, in our hands ssp. *zebrina* (Cytotype II) was clearly distinguishable from ssp. *malaccensis* (Cytotype I) based on their mitochondrial genome. Consequently, molecular data based on nuclear as well as on cytoplasmic genomes will be appropriate for elucidation of genetic relationship of the present day subspecies; however, to reach precise and conclusive classification the analysis of more genotypes will be necessary.

On the other part MST analysis of the haplotypes revealed the presence of primary centres of origin in both chloroplast and mitochondrial haplotypes indicating common origin of these cytoplasmic genomes. Therefore, our findings support the idea of Horry and Jay (1988) who suggested the existence of an ancient *acuminata* gene-pool as a source of the latter subspecies or varieties, however based on our results an ancient gene-pool may as well be suggested for *M. balbisiana*.

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