Microsatellite markers for genome analysis in *Musa* and *Mycosphaerella*

D. Kaemmer[1], C. Neu[1], G. Farashahi[1], R.L. Jarret[2]
A. James[3], R. Swennen[4], C. Pasberg-Gauhl[5]
F. Gauhl[5], D. Fischer[6], G. Kahl[1] & K. Weising[1]

[1]Plant Molecular Biology, Biocentre
University of Frankfurt/Main, Germany

[2]Plant Genetic Resources, USDA
Griffin, Georgia, U.S.A.

[3]Centro de Investigacion Cientifica de Yucatan
Merida, Mexico

[4]Laboratory of Tropical Crop Improvement
Katholieke Universiteit Leuven, Belgium

[5]International Institute of Tropical Agriculture
Onne Station, Nigeria

[6]Institute of Plant Genetics and Crop Plant Research
Gatersleben, Germany

1. Introduction

British scientists have produced a clear and coherent classification system, based on extensive collection and breeding efforts to characterize the diversity of the genus *Musa* (Cheesman, 1947-50; Simmonds & Shepherd, 1955; Simmonds, 1962). First attempts of numerical taxonomy lead to the development of a system of agro-morphological descriptors serving breeders world-wide as markers (Simmonds & Weatherup, 1990; IPGRI-INIBAP/CIRAD, 1996). Isoenzyme patterns successfully distinguished *M. acuminata* subspecies and identified the B genome of *M. balbisiana* in interspecific hybrids (Jarret & Litz, 1986). However, nuclear restriction fragment length polymorphism (RFLP) markers have been shown to be superior with respect to their reliability and have therefore been preferred for molecular taxonomy (Carreel *et al.*, 1994) and low density mapping (Fauré *et al.*, 1993). Phylogenetic questions have also been addressed using RFLP markers of chloroplasts and mitochondria (Gawel & Jarret, 1991), and maternal inheritance of chloroplasts as well as paternal inheritance of mitochondria has been demonstrated (Fauré *et al.*, 1994).

A number of locus-specific microsatellites, also called simple sequence repeat (SSR) or sequence-tagged microsatellite site (STMS) markers were recently developed for *Musa* genome analysis (Kaemmer *et al.*, 1997; Crouch *et al.*, 1998; Lagoda *et al.*, 1998). These powerful markers were used to establish anchor maps, perform marker-assisted selection, identify cultivars, and infer phylogenetic relationships. Although increasing numbers of microsatellite markers became available, at least one additional, more cost-effective marker system is necessary to saturate genetic maps, enable the identification of closely related cultivars, and screen larger parts of the genome for mutations. PCR-based genome scanning techniques such as random amplified polymorphic DNA (RAPD) or DNA amplification fingerprinting (DAF) were successfully used in *Musa* genome characterisation, but suffer from limited reproducibility between different laboratories (Kaemmer *et al.*, 1992; Newbury & Ford-Lloyd, 1993; Bhat *et al.*, 1995). The method of choice to obtain reproducible high resolution DNA scanning data seems to be the amplification fragment length polymorphism (AFLP) technique (Vos *et al.*, 1995). This technique combines DNA restriction fragmentation and specific DNA amplification using 18-30 bp long pairs of partially degenerated oligonucleotides with subsequent analysis of PCR products by polyacrylamide gel electrophoresis, and has been successfully applied for the detection of polymorphisms associated with somaclonal variation (Engelborghs *et al.*, 1998).

Since microsatellites are also excellent DNA markers for population biology of fungi, three partial genomic libraries were produced to isolate SSR-containing clones from *Mycosphaerella fijiensis*, the causal agent of black leaf streak disease. Eleven out of 28 designed primer pairs were useful for an initial study of *M. fijiensis* from Nigeria and Mexico. The results presented in this paper will help to better understand the genetic diversity of this *Musa* pathogen and to optimize future collection activities.

2. Materials and Methods

Musa genotypes used for this study are listed in Tables 1 and 4. The following PCR conditions (PERKIN ELMER 9600™) were used to amplify different STMS loci (Table 2): 94°C for 1 minute, 35 cycles of [94°C for 15 seconds, 55°C for 30 seconds, 72°C for 45 seconds], 72°C for 5 minutes.

Reaction volumes varied between 10 and 50 µl using 1 ng/µl template DNA. Final concentrations in the reaction were 1 µM each primer, 0.2 mM dNTPs, 2.2 mM $MgCl_2$, and 0.1 U/µl Goldstar™ Taq polymerase (EUROGENTECH). PCR products were separated on sequencing gels and stained with silver. Alternatively, α^{32}PdCTP was included in the PCR, and gels were autoradiographed.

Table 1. *Musa* genotypes used to study microsatellite markers

No.	Name	Genome	Classification	Status
1	*M. acuminata* ssp. *malaccensis*	AA	ITC 0250	wild
2	PISANG LILIN	AA		cultivar
3	*M. acuminata* ssp. *burmannicoides*	AA	Type Calcutta IV	wild
4	PISANG MAS	AA		cultivar
5	NIYARMA YIK	AA		cultivar
6	SH-3362	AA	FHIA	cultivar
7	SH-3142	AA	FHIA	cultivar
8	*M. balbisiana*	BB	Type Tani	wild
9	*M. ornata*	2n=22		wild
10	*M. abaca*	2n=20		wild
11	*Ensete* sp.	2n=18		wild
12	HIGHGATE	AAA	Gros Michel	cultivar
13	GRANDE NAÏNE	AAA	Cavendish	cultivar
14	DWARF PARFITT	AAA	Cavendish	cultivar
15	WILLIAMS	AAA	Cavendish	cultivar
16	MAGHARABI	AAA		cultivar
17	TAFETAN VERDE	AAA		cultivar
18	HINDI	AAA		cultivar
19	ROJO	AAA		cultivar
20	TAIWAN	AAA		cultivar
21	SH-3436	AAAA	SH-3362 x HIGHGATE	tetraploid hybrid
22	AVP-67	AAB	plantain	cultivar
23	FRENCH REVERSION	AAB	plantain	cultivar
24	OBINO L'EWAI	AAB	plantain	cultivar
25	BOBBY TANNAP	AAB	plantain	cultivar
26	wrong classification as HARTON	ABB	cooking banana	cultivar
27	PELIPITA	ABB	cooking banana	cultivar
28	SABA	ABB	cooking banana	cultivar

Table 2. Microsatellite loci used to study *Musa* genetic diversity.

Name	Microsatellite	Primer Sequences	Length of Sequenced Allele
MaSSR-07	$(CT)_9$	5'-AAGAAGGCACGAGGGTAG-3' 5'-CGAACCAAGTGAAATAGCG-3'	212 bp
MaSSR-08	$(AG)_{12}$	5'-GGAAAACGCGAATGTGTG-3' 5'-AGCCATATACCGAGCACTTG-3'	250 bp
MaSSR-18	$(GAA)_{10}$	5'-CGTCACAGAAGAAAGCACTTG-3' 5'-CCTCTCCATCGTCATCAATC-3'	179 bp
MaSSR-24	$(CT)_{11}$	5'-GAGCCCATTAAGCTGAACA-3' 5'-CCGACAGTCAACATACAATACA-3'	172 bp

Table 3. Hierarchical samples of *Mycosphaerella fijiensis* isolates from Nigeria and Mexico

Set-No.	Identification Code	Hierarchical Level	Location	Host Cultivar
B1	Mf-Nig		Mfamosing	Agbagba
B2	Mf-Nig		Umoghun	Agbagba
B3	Mf-Nig 639	Plant 1	Igbofia	Agbagba
B4	Mf-Nig 653	Plant 2	Igbofia	Agbagba
B5	Mf-Nig 642	Plant 6	Igbofia	Agbagba
B6	Mf-Nig 645	Plant 7	Igbofia	Agbagba
B7	Mf-Nig 670	Plant 9, Leaf 8	Igbofia	Agbagba
B8	Mf-Nig 613	Plant 9, Leaf 10	Igbofia	Agbagba
B9	Mf-Nig 634	Plant 9, Leaf 11	Igbofia	Agbagba
B10	Mf-Nig 638	Plant 9, Leaf 12	Igbofia	Agbagba
B11	Mf-Nig 621	Plant 9, Leaf 10, Lesion 99	Igbofia	Agbagba
B12	Mf-Nig 622	Plant 9, Leaf 10, Lesion 99	Igbofia	Agbagba
B13	Mf-Nig 626	Plant 9, Leaf 10, Lesion 99	Igbofia	Agbagba
B14	Mf-Nig 627	Plant 9, Leaf 10, Lesion 99	Igbofia	Agbagba
C1	Mf-Mex 004		Yucatan	Williams
C2	Mf-Mex 010		Chiapas	Grande Naine
C3	Mf-Mex 020		Tabasco	Grande Naine
C4	Mf-Mex 058	Plant 1	Colima	Grande Naine
C5	Mf-Mex 030	Plant 2	Colima	Grande Naine
C6	Mf-Mex 031	Plant 3	Colima	Grande Naine
C7	Mf-Mex 036	Plant 5, Leaf 2	Colima	Grande Naine
C8	Mf-Mex 039	Plant 5, Leaf 3	Colima	Grande Naine
C9	Mf-Mex 037	Plant 5, Leaf 4	Colima	Grande Naine
C10	Mf-Mex 038	Plant 5, Leaf 5	Colima	Grande Naine
C11	Mf-Mex 032	Plant 4, Leaf 4, Lesion 1	Colima	Grande Naine
C12	Mf-Mex 033	Plant 4, Leaf 4, Lesion 1	Colima	Grande Naine
C13	Mf-Mex 034	Plant 4, Leaf 4, Lesion 1	Colima	Grande Naine
C14	Mf-Mex 035	Plant 4, Leaf 4, Lesion 1	Colima	Grande Naine

M. fijiensis isolates were hierarchically sampled from banana plantations in Nigeria and Mexico, followed by single-sporing and *in vitro* culture. DNA was isolated from mycelia using a Fast DNA Kit (Bio 101). PCR conditions for the amplification of *Mycosphaerella fijiensis* SSR markers have been published (Neu *et al.*, 1999). Table 3 describes the used *M. fijiensis* isolates. PCR products were separated on a sequencing gel and autoradiographed. Primer sequences of *M. fijiensis* SSRs are shown in Table 5.

3. Results

3.1. Genetic diversity of microsatellite markers in wild and cultivated Musa

Eleven microsatellite loci were investigated using a set of 28 *Musa* genotypes described in polymorphism information content (0.85) and a mean heterozygosity value of 0.60 (Fischer, 1996). The results obtained with MaSSR-24 demonstrate that this particular locus can be amplified in all studied Eumusa genotypes but not in the representatives of Rhodochlamys, Australimusa and Ensete (lane 9-11; Fig. 1).

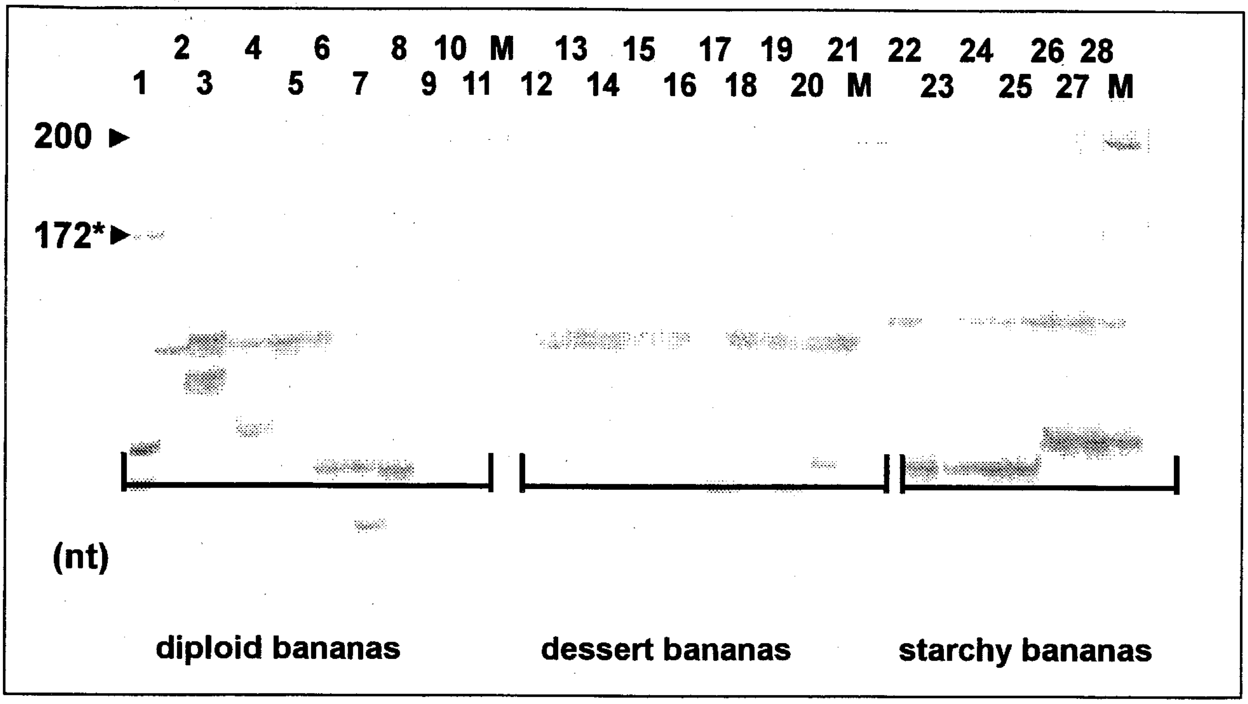

Fig. 1. PCR products of MaSSR-24 locus in 28 *Musa* genotypes (Tab. 1). PCR fragments were separated in a denaturing polyacrylamide gel and subsequently stained with silver nitrate (Fischer 1996); * sequenced allele; M = 200 nt fragment of 100 bp DNA ladder.

Table 1. wild and cultivated diploid genotypes showed a high polymorphism information content (0.85) and a mean heterozygosity value of 0.60 (Fischer, 1996). The results obtained with MaSSR-24 demonstrate that this particular locus can be amplified in all studied Eumusa genotypes but not in the representatives of Rhodochlamys, Australimusa and Ensete (lane 9-11; Fig. 1). Out of the eight diploid genotypes amplifying this locus six are heterozygous and only two are homozygous (PISANG LILIN and *M. balbisiana*). The wild banana CALCUTTA IV is heterozygous at this locus but homozygous at other loci, e.g. MaSSR-07 (Fischer, 1996). Three of the nine AAA dessert banana cultivars possess deviating alleles (TAFETAN VERDE, ROJO and TAIWAN, lanes 17, 20, 21; Fig. 1) distinguishing them from the other cultivars, mainly from the Cavendish group. The dominant allele of the other edible cultivars is also shared by all edible diploid but not the wild bananas, except for one allele which is shared also by CALCUTTA IV (Fig. 1). Interestingly, the allele from the A genome(s) of the interspecific hybrids (AAB, ABB) is different and is obviously derived from a diploid ancestor not present in this set (presumably *M. acuminata* ssp. *banksii*). The size of the allele from *M. balbisiana* type TANI seems to be identical to that carried by all the plantains (AAB), but different from the allele of all the cooking banana types (ABB).

3.2. Transferability of selected microsatellite markers within the familiy of Musaceae

The transferability of *M. acuminata* ssp. *malaccensis* microsatellite markers to other *Musa* species as well as to the second genus of the Musaceae family (*Ensete*) and one representative of the related Strelitziaceae was investigated at four selected loci (Tab. 4). Only one locus could be amplified in all 28 genotypes (MaSSR-01). A second locus (MaSSR-07) could be detected in all members of the genus *Musa*, but not in *Ensete* and *Ravenala madagascarensis* (Strelitziaceae). The third locus (MaSSR-08) is not even species-specific because one plantain (OSOABOASO) and 14 banana cultivars from the Philippines (Hautea *et al.*, this issue) did not amplify a PCR product. This particular locus can be amplified in other

Eumusa species but not in *M. balbisiana* and *M. basjoo*. All investigated Rhodochlamys species and two of the four Australimusa species produced a DNA fragment but not *M. beccarii* and *Ensete*. MaSSR-18 can distinguish species of all *Musa* sections.

Table 4. Transferability of *M. acuminata* ssp. *malaccensis* locus-specific microsatellite markers to different taxa of the Musaceae. + Product present; - no product

No.	Cultivar/Species	Genome/Section	MaSSR-01	MaSSR-07	MaSSR-08	MaSSR-18
1	NTANGA	AAB/Eumusa	+	+	+	+
2	BOBBY TANNAP	AAB/Eumusa	+	+	+	+
3	3 VERT	AAB/Eumusa	+	+	+	+
4	OSOABOASO	AAB/Eumusa	+	+	-	+
5	BIG EBANGA	AAB/Eumusa	+	+	+	+
6	IHITISIM	AAB/Eumusa	+	+	+	+
7	*M. acuminata* ssp. *malaccensis*	AA/Eumusa	+	+	+	+
8	*M. acuminata* ssp. *microcarpa*	AA/Eumusa	+	+	+	+
9	*M. acuminata* ssp. *truncata*	AA/Eumusa	+	+	+	+
10	*M. acuminata* ssp. *siamea*	AA/Eumusa	+	+	+	+
11	*M. acuminata* ssp. *zebrina*	AA/Eumusa	+	+	+	-
12	*M. balbisiana*	BB/Eumusa	+	+	-	+
13	*M. balbisiana*	BB/Eumusa	+	+	-	+
14	*M. balbisiana*	BB/Eumusa	+	+	-	+
15	GRANDE NAINE	AAA/Eumusa	+	+	+	+
16	*M. basjoo*	Eumusa	+	+	-	+
17	*M. schizocarpa* Nr. 1	Eumusa	+	+	+	+
18	*M. ornata*	Rhodochlamys	+	+	+	+
19	*M. velutina*	Rhodochlamys	+	+	+	-
20	*M. laterita*	Rhodochlamys	+	+	+	-
21	*M. textilis*	Australimusa	+	+	+	-
22	*M. pekelii* Type angustigema	Australimusa	+	+	+	+
23	*M. maclay*	Australimusa	+	+	-	-
24	*M. lolodensis*	Australimusa	+	+	+	-
No.	Cultivar/Species	Genome/Section	MaSSR-01	MaSSR-07	MaSSR-08	MaSSR-18
25	*M. pekelii* Type pekelii	Australimusa	+	+	-	+
26	*M. beccarii*	Callimusa	+	+	-	-
27	*Ensete* sp.	Musaceae	+	-	-	+
28	*Ravenala madagascarensis*	Strelitziaceae	+	-	-	-

3.3. Comparison of M. fijiensis genotypes from Nigeria and Mexico

Fourteen hierarchically sampled isolates each from Nigeria and Mexico (Tab. 3) have been genotyped using 11 SSR loci (Tab. 5). A typical result is shown in figure 2. Although most of the genetic variability was found in the isolates sampled from different locations and different plants, respectively, even the isolates collected from one and the same lesion and therefore originating from the same cross of two parental isolates could be distinguished by their different unique haplotypes. The haplotype of a given isolate was obtained from the combination of the allele sizes of only eleven microsatellite loci. The allele sizes were calculated from a sequencing ladder (known sequence of M13 phage) run side by side with the fungal DNA fragments. Up to seven different alleles could be found in the populations from the two continents. In most cases the predominant allele of Nigerian isolates was different from the one of Mexican isolates though overlapping allele sizes occured.

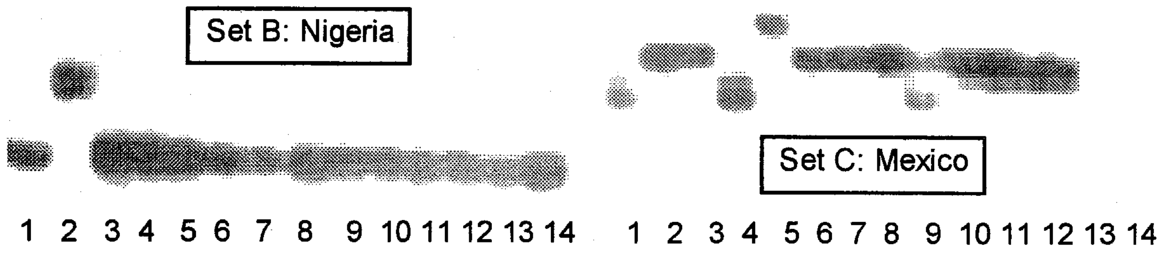

Fig. 2. Radioactively labled PCR fragments of *M. fijiensis* locus MfSSR-203 (Tab. 5) amplified from 28 different isolates (Tab. 3) resolved by denaturing polyacrylamide gel electrophoresis.

In case of MfSSR-203 the predominant alleles from Nigeria and Mexico differed by 12 bp or 4 units of the trinucleotide microsatellite. This might be an indication for the genetic differences present in the different founder populations entering the American (Honduras 1972) and the African continent (Zambia 1973) from Southeast Asia. Almost all isolates were haploid because only a single PCR fragment per locus could be detected. However, the DNA of isolate C-9 of Figure 2 amplified two bands corresponding to two different alleles. These alleles might either belong to two different loci originating from a duplication of the originally single microsatellite locus, or derive from two independent isolates which were co-isolated during the single-sporing procedure.

Table 5. PCR primer sequences and characteristics of *M. fijiensis* locus-specific microsatellites

Locus	Repeat	Primer sequences (5′ - 3′)	Expected size (bp)	Numbers of alleles			Allelic diversity	
				Nig	Mex	Total	Nig	Mex
*Mf*SSR-005	$(CAACACA)_4$	TCCAAATTCCATCGTTGTCA CGATGATTTGGGTGGTCAAGCTA	158	1	3	4	0.00	0.57
*Mf*SSR-018	$(GAA)_9$	TAGTGCGAGTAGCGAGGCAG GACCTAAAGGCAATAGGGCTT	104	1	1	2	0.00	0.00
*Mf*SSR-025	$(CA)_{18}$	CATGACTGACGTCCTCTTCTCA ATATGGGAAGGGGAAAGGTG	176	3	2	5	0.28	0.41
*Mf*SSR-058	$(TG)_7G_3(TG)_9$	TTCGCAAAAAGTCCTTCAGC GATGGAGGCACGAAAAGGTA	166	1	2	3	0.00	0.25
*Mf*SSR-061	$(CAA)_8$	TGCAAACTCTGATGCTGGAC TTCAGAGGCTCGTCTTTGGT	124	1	3	4	0.00	0.57
*Mf*SSR-137	$(GT)_{19}$	GGCTCGAAGTGGACTAGCAC CTGGTCGAGGGTCGGG	243	3	3	5	0.42	0.52
*Mf*SSR-145	$(GT)_{19}$	GATGAGAAGGATCTCGTCGG GGCTCGAAGTGGACTAGCAC	181	2	3	5	0.39	0.38
*Mf*SSR-175	$(CA)_{16}(CT)_{13}(CA)_{27}$	AACCTCACATAGGCTGCCAC TATACCTTTCGTTCGGCCTG	286	3	4	7	0.49	0.60
*Mf*SSR-194	$(GTT)_{10}$	CATCTTTGAGGAGGCAAAGC AGATTCCTTAGGCGGCATTT	294	3	2	5	0.55	0.41
*Mf*SSR-203	$(GTT)_7$	CTCTGTGGCGTAAGTGGGTG TGATTGCACAGCAGGAAGAG	227	2	3	5	0.13	0.44
*Mf*SSR-244	$(TG)_{29}$	GGCCATTTCATTTGCAAGAC ATGCCACAAAATCTCCATCC	215	3	2	5	0.38	0.38

4. Discussion

Microsatellite markers developed for *Musa* genome analysis can be used to trace back the potential origin of hybrid cultivars from cultivated and/or wild ancestors. After hybridization of two *M. acuminata* subspecies (AA) leading to a triploid, almost sterile cultivar (AAA), the genomic constitution is fixed and will not be changed by vegetative propagation. However, somatic mutations or somaclonal variations might have major effects on the phenotype altering typical characters originating from the parents. This might also cause confusion in the systematics of cultivars, especially if they carry different names (Swennen, 1985). Depending on the degree of outcrossing, geographic isolation, and the influence of other evolutionary forces, the allelic diversity at microsatellite loci of diploid germplasm may change over long times. To overcome the problem of homoplasy in the overlapping distributions of microsatellite alleles of different (sub)species, sequencing of alleles is mandatory to trace back ancestor alleles of hybrids which might be found in genotypes of existing collections. The different origin of plantains and cooking bananas is not only evident from Fig. 1, but also witnessed by other microsatellite markers, *e. g.* MaSSR-01 (Kaemmer *et al.*, 1997). Since BBB cultivars have never been observed, cooking banana cultivars (ABB) must have developed via an AB intermediate. On the other hand, plantain cultivars (AAB) could have originated directly from a non-reduced diploid megagamete (AA) and a haploid *M. balbisiana* microgamete (B). This implies not only different *M. acuminata* but also different *M. balbisiana* parents for both types of interspecific hybrids. In future, we are interested to investigate the allelic constitution of different AB hybrids in comparison with ABB cultivars.

The rapid spray of Black Sigatoka throughout the banana-growing tropical world in the second half of this century emphasizes the need to better understand the epidemiology and genetics of *M. fijiensis*. The newly developed microsatellite markers not only allow population studies, but also genetic mapping, and the correlation between phenotypes and genotypes. The present data support the view of a predominant sexual propagation of *M. fijiensis* in both Nigeria and Mexico with concomitant high recombination rates between different alleles. This necessarily implies that genes responsible for pathogenicity and virulence are also permanently recombined to increase the fitness of the pathogen under changing environmental conditions, e. g. their host plant genotypes.

5. Acknowledgements

Research of the authors has been funded by German Research Council (DFG Ka 332/15), European Community (ERB-IC-18/CT-970192), International Atomic Energy Agency (CRP 302-02-GFR-8148), and Vereinigung von Freunden und Förderern der Johann Wolfgang Goethe-Universität Frankfurt am Main. D. Kaemmer and K. Weising appreciate an invitation of IAEA to the meetings of this CRP.

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