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Chloroplast DNA restriction fragment length polymorphisms (RFLPs) in *Musa* species

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Summary. Taxonomic and phylogenetic determinations within the genus *Musa* are established using a numerical, morphology-based scoring system. However, within this system, the classification and relationships of some types are disputed. The application of chloroplast DNA (cpDNA) restriction fragment length polymorphism (RFLP) analysis to *Musa* taxonomy provided valuable, supplemental information about the classification of, and relationships between, *Musa* species and subspecies. Whole-cell DNA was extracted from lyophilized *Musa* leaf-blade tissue and digested with various restriction enzymes, Southern blotted onto nylon membranes, and probed using radioactively labeled heterologous orchid cpDNA fragments. Phylogenies were inferred from cpDNA RFLP patterns using PAUP software. The relationships between most species examined were as expected; however, some species (*M. beccarii* and *M. basjoo*) did not conform to the conventional morphology-based phylogeny.

Key words: Banana – cpDNA – Plantain – Systematics

Introduction

The genus *Musa* is composed of four sections; section Eumusa contains the bananas and plantains, the principle carbohydrate source for over 100 million people worldwide (Rowe 1981). All bananas and plantains (except the Fe'i bananas) are derived from *Musa acuminata* Colla. or hybrids of *M. acuminata* with *M. balbisiana* Colla. Due to the occurrence of interspecific hybrids and their often triploid nature, initial attempts at taxonomic classification of individual banana and plantain clones were unclear and often ambiguous (Simmonds and Shepherd 1955). A categorization system was developed based upon the numerical scoring of morphological characteristics (Simmonds and Shepherd 1955). This system determines the relative genetic contribution of *M. acuminata* (A genome) and/or *M. balbisiana* (B genome), as well as ploidy level. However, the classification of some types has been disputed. Morphology-based classifications have also been used for the purpose of inferring phylogenies of species and subspecies within *Musa* (Simmonds and Weatherup 1990).

Difficulties in classifying individual clones to a particular genomic grouping, i.e., AAA, AAB, ABB, etc., may be due to the high incidence of somatic mutations within specific clones (Simmonds 1962; Vuylsteke et al. 1988) or are a result of the confounding influences of environmental or maternal (cytoplasmic) effects on the whole plant phenotype (Jarret and Litz 1986a). These influences may result in an over- or underestimation of the extant diversity and may confound efforts to infer phylogenies based upon morphological characteristics.

The limitations of the current classification system suggest that a classification scheme based upon molecular, rather than morphological, markers could provide more accurate assessments of diversity and systematic relationships between *Musa* species and clones. Isozyme polymorphisms have been utilized for clonal identification and for quantification of genetic diversity in *Musa* (Bonner et al. 1974; Jarret and Litz 1986b; Rivera 1983). However, the number of isozymes available for analysis frequently limits their usefulness as markers. The use of nucleic acid probes as molecular markers to detect polymorphisms in base sequences is a preferred alternative to the analysis of enzyme polymorphisms – because of the potentially unlimited number of probe/enzyme combinations available.

In this paper, we demonstrate the use of cpDNA RFLP analysis to differentiate between various *Musa* species and subspecies, and to infer phylogenies between these species and subspecies.

Materials and methods

Plant materials were obtained from Dr. P. Rowe, Honduran Foundation of Agricultural Investigation (FHIA), La Lima, Honduras, and through the International Network for the Improvement of Bananas and Plantains (INIBAP) germplasm transit center at the Catholic University of Louvain (KUL), Belgium. Plant material received in vitro was recultured onto Murashige and Skoog (1962) media in 25 × 150 mm glass culture tubes and grown to a height of 6–8 cm before being transferred to soil.

Blade tissue from furled or recently expanded leaves was frozen and lyophilized. Whole-cell DNA was extracted essentially as described by Murray and Thompson (1980). Lyophilized leaf tissue (500–1,000 mg) was ground in liquid N and added to 15 ml of extraction buffer [2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 1.4 *M* NaCl, 20 m*M* EDTA, and 0.1% (v/v) mercaptoethanol]. The extraction mixture was mixed by inversion and incubated at 65 °C for 60 min. Fifteen milliliters of chloroform : isoamyl alcohol (24 : 1, v/v) was added, the mixture was shaken gently for 15 min at room temperature, then centrifuged at 6,500 × *g* for 5 min. The aqueous phase was removed and filtered through miracloth (CalBiochem). DNA was precipitated from the aqueous phase by the addition of an equal volume of ice-cold isopropanol. The DNA was removed using a glass hook, washed in 70% EtOH, and resuspended in TE [10 m*M* Tris (pH 8.0), 1 m*M* EDTA (pH 8.0)] (Saghai-Maroof et al. 1984). DNA solutions were treated with RNAse (10 µg/ml) for 30 min at room temperature, ethanol precipitated (Maniatis et al. 1982), and resuspended in 400 µl TE. Yields ranged from 100 to 500 µg (OD_{260}) per gram of fresh weight leaf tissue.

DNA extracts (≈8 µg) from each clone were digested with EcoRI, HindIII, BamHI, MspI, HapII, CfoI, or SacI according to the manufacturer's recommendations for 12 h at 37 °C. Fragments were separated on 0.8% agarose gels (2 V/cm) for 12 h, transferred to nylon membranes (Biotrans) according to Southern (1975), and baked in a vacuum oven at 80 °C for 1 h.

Membranes were prehybridized for 3–4 h in 5 × SSC, 50 m*M* NaH_2PO_4, 5 × Denhardt's solution, 2.5 m*M* EDTA, 0.4% SDS, 5% dextran sulfate, and 0.001% sonicated, denatured salmon sperm DNA. Denatured, ^{32}P-labeled DNA probes were added to the prehybridization mixture and allowed to hybridize overnight. DNA probes were ^{32}P-labeled with a random primer DNA labeling system (Bethesda Research Laboratories, Cat. No. 8187SA). Membranes were washed for 20 min each in 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS; they were then covered with plastic wrap and exposed to X-ray film at −30 °C, as described in detail by Gawel and Jarret (1991).

Probes used in this study were isolated from orchid (*Oncidium excavatum*) chloroplast DNA (cpDNA), generously provided by Dr. M. Chase, University of North Carolina. Probes used included: 9d, 6b, 10a, 11, 12b, 16, and 18a. Entire plasmids (containing heterologous sequence inserts) were labeled and used as probes. Fragment positions on autoradiographs were processed into 1-0 matrices and analyzed with PAUP software (Swofford 1985). PAUP options used were SWAP = ALT, HOLD = 25, and MULPARS (max. = 100). The CONTREE program of PAUP was used to construct an Adams consensus tree from all the equally parsimonious trees generated by PAUP.

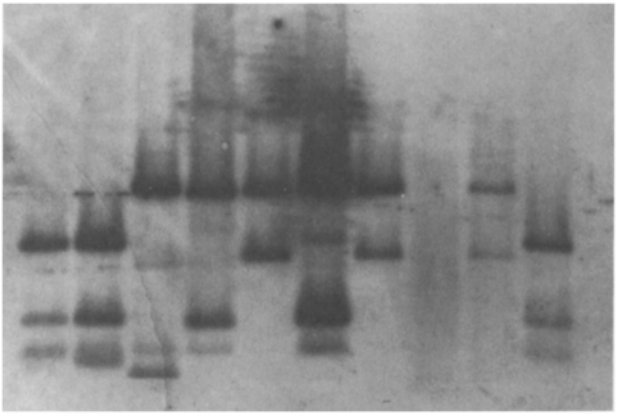

Fig. 1. Autoradiograph (HapII digest, probe 9d) of (*left* to *right*): *M. balbisiana*, *M. ornata*, *M. coccinea*, *M. basjoo*, *M. acuminata* ssp. *truncata* (FHIA), *M. velutina*, *M. acuminata* ssp. *malaccensis*, *M. beccarii*, *M. acuminata* spp. *truncata* (KUL), and *M. balbisiana*

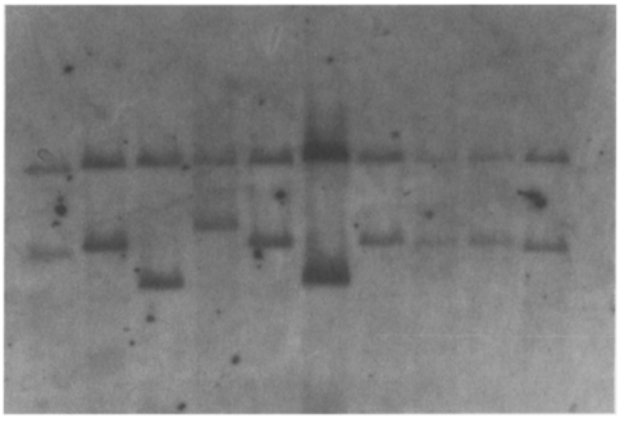

Fig. 2. Autoradiograph (MspI, probe 18b) of (*left* to *right*): *M. balbisiana*, *M. ornata*, *M. coccinea*, *M. basjoo*, *M. acuminata* spp. *truncata* (FHIA), *M. velutina*, *M. acuminata* ssp. *malaccensis*, *M. beccarii*, *M. acuminata* ssp. *truncata* (KUL), and *M. balbisiana*

Table 1. *Musa* species examined for cpDNA RFLPs

Species	Section	n=
M. balbisiana Colla.	Eumusa	11
M. basjoo Sieb.	Eumusa	11
M. beccarii Simmonds	Callimusa	9
M. coccinea Andr.	Callimusa	10
M. ornata Roxb.	Rhodochlamys	11
M. textilis Nee.	Australimusa	10
M. velutina Wendl. & Drude	Rhodochlamys	11
M. acuminata ssp. *banksii* Simmonds	Eumusa	11
M. acuminata ssp. *burmannica* Simmonds	Eumusa	11
M. acuminata ssp. *malaccensis* Simmonds	Eumusa	11
M. acuminata ssp. *microcarpa* Simmonds	Eumusa	11
M. acuminata ssp. *siamea* Simmonds	Eumusa	11
M. acuminata ssp. *truncata* Simmonds	Eumusa	11

Table 2. Manhattan character difference matrix for *Musa* clones examined

	M. balbisiana	*M. ornata*	*M. coccinea*	*M. basjoo*	*M. acuminata* ssp *truncata*[a]	*M. velutina*	*M. acuminata* ssp. *malaccensis*	*M. beccarii*	*M. acuminata* ssp. *truncata*[b]	*M. acuminata* ssp. *siamea*[a]	*M. acuminata* ssp. *microcarpa*	*M. acuminata* ssp. *banksii*	*M. acuminata* ssp. *burmannica*	*M. acuminata* spp. *siamea*[b]	*M. textilis*
M. balbisiana	0.000														
M. ornata	0.340	0.000													
M. coccinea	0.450	0.510	0.000												
M. basjoo	0.340	0.360	0.430	0.000											
M. acuminata ssp. *truncata*[a]	0.450	0.330	0.480	0.330	0.000										
M. velutina	0.350	0.330	0.320	0.270	0.240	0.000									
M. acuminata ssp. *malaccensis*	0.430	0.310	0.480	0.350	0.020	0.240	0.000								
M. beccarii	0.412	0.287	0.500	0.312	0.075	0.275	0.050	0.000							
M. acuminata ssp. *truncata*[b]	0.420	0.300	0.510	0.340	0.050	0.270	0.030	0.050	0.000						
M. acuminata ssp. *siamea*[a]	0.424	0.293	0.485	0.323	0.051	0.253	0.030	0.051	0.020	0.000					
M. acuminata ssp. *microcarpa*	0.424	0.313	0.465	0.343	0.091	0.293	0.071	0.101	0.061	0.040	0.000				
M. acuminata ssp. *banksii*	0.420	0.340	0.470	0.340	0.110	0.290	0.090	0.125	0.080	0.061	0.020	0.000			
M. acuminata ssp. *burmannica*	0.434	0.303	0.495	0.333	0.061	0.263	0.040	0.051	0.030	0.010	0.051	0.071	0.000		
M. acuminata spp. *siamea*[b]	0.418	0.306	0.500	0.347	0.071	0.276	0.051	0.051	0.020	0.021	0.041	0.061	0.031	0.000	
M. textilis	0.295	0.477	0.295	0.318	0.386	0.318	0.386	0.417	0.409	0.364	0.364	0.364	0.386	0.409	0.000

[a] Clone obtained from FHIA, La Lima, Honduras
[b] Clone obtained from germplasm transit center at the Catholic University of Louvain (KUL), Belgium

Results

Species, subspecies, section, and number of chromosomes of each clone examined are presented in Table 1. Not every probe/restriction enzyme combination produced discernable polymorphisms between the clones examined. Representative autoradiographs are shown in Figs. 1 and 2.

The character difference matrix produced by PAUP is presented in Table 2. Data from the 49 probe/enzyme combinations led to the calculation of 74 equally parsimonious trees by PAUP. Most of the differences between trees were due to rearrangements between and within the *M. acuminata* subspecies complex. The consensus cladagram (Fig. 3) produced from the cpDNA RFLP data illustrates the differences in cpDNA between and within *Musa* species.

The branching pattern in Fig. 3 illustrates that the *M. acuminata* subspecies behaved as expected, i.e., they are closely clustered. Within this cluster, however, cpDNA variability is evident both between and within the subspecies; two different clones each of *M. acuminata* ssp. *truncata* and *M. acuminata* ssp. *siamea* were examined, neither of which had identical cpDNA. Intrasubspecific variability has also been documented using morphological characteristics (Simmonds 1966). Figure 3 also shows the placement of *M. acuminata* ssp. *banksii* within the *M. acuminata* subspecies complex. The placement supports the assertion that *M. acuminata* spp. *banksii* should be classified as a subspecies of *M. acuminata* and not as separate species, as suggested by Argent (1976).

M. balbisiana and *M. textilis* belong to different sections and have different chromosome numbers. Despite these differences, these species have enough genetic similarity to readily hybridize to produce the fiber-yielding clone 'Canton' (Simmonds 1962), and enough morphological similarity to be classified as closely related (Simmonds and Weatherup 1990). These similarities are reflected in the cytoplasmic relationship depicted in Fig. 3. Our analysis shows the cytoplasm *M. textilis* to be very close to that of *M. balbisiana*, reflecting the close relationship of these species.

Numerical taxonomic analysis (Simmonds and Weatherup 1990) places *M. basjoo* much closer to the *M. acuminata* subspecies complex than does the cpDNA-based analysis. Again, this discrepancy may be due to the basis of classification (morphological versus cytoplasmic characteristics). The relative placements of the remaining species, *M. ornata*, *M. coccinea*, and *M. velutina*, are in general agreement with those based upon morphological characteristics (Simmonds and Weatherup 1990).

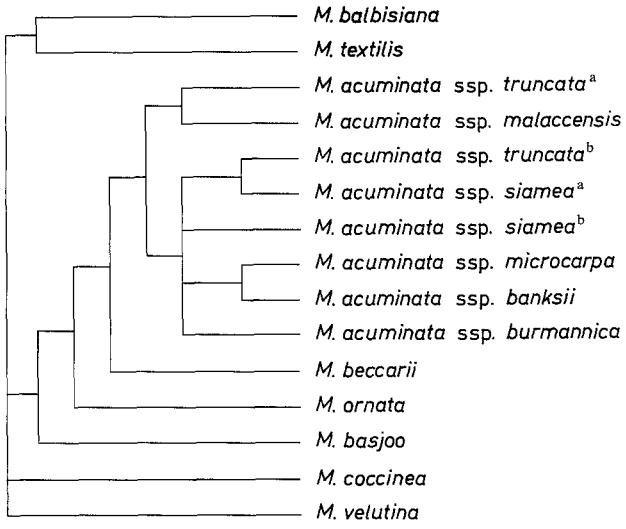

Fig. 3. Consensus cladagram of *Musa* species and *M. acuminata* subspecies based upon cpDNA RFLP data
[a] Clone obtained from FHIA, La Lima, Honduras
[b] Clone obtained from germplasm transit center at the Catholic University of Louvain (KUL), Belgium

Discussion

Most of the relationships depicted in Fig. 3 agree with those based upon morphological data (Simmonds and Weatherup 1990). Others, however, do not. One explanation for this discrepancy may be that our cladagram is based exclusively upon cpDNA-derived data, while morphological characteristics are based on the expression of nuclear genes and nuclear × cytoplasmic gene interactions.

Although classified as a 'relic species' (Simmonds 1962), the cytoplasm of *M. beccarii* was determined to be more like that of the *M. acuminata* subspecies complex that the other species examined (Fig. 3). Morphologically, *M. beccarii* and *M. acuminata* are very distinct, they have different basic chromosome numbers (n = 9 and n = 10, respectively), are believed to have evolved along different paths, and are not known to hybridize (Simmonds 1962). The similarity in cpDNA could be due to a common ancestor, or perhaps *M. beccarii* is the aneuploid product of an interspecific hybridization. Shepherd's (1959) documentation of multivalent formation in meiosis of *M. beccarii* supports this hypothesis.

The data presented here demonstrate the use of cpDNA RFLPs for phylogenetic analysis of *Musa* species and subspecies. With a few exceptions, the results were in general agreement with previously published data. Some species, e.g., *M. beccarii* and *M. basjoo*, were classified quite differently than expected, suggesting the need for further investigation. Perhaps the use of a greater number of probe/enzyme combinations or the analysis of nuclear or mitochondrial DNA diversity would help clarify these relationships.

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