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Physical mapping of the 18S-25S and 5S ribosomal RNA genes in diploid bananas

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Abstract

Fluorescent *in situ* hybridisation (FISH) was used to determine the number and distribution of the 18S-25S and 5S rDNA sites on mitotic chromosomes of 6 wild and 2 edible diploid (2n=22) accessions belonging to the two banana species, *Musa acuminata* and *M. balbisiana*. FISH with the 18S-25S probe resulted in signals on one pair of chromosomes, the position of signals corresponded to the secondary constriction at the end of a short arm. The intensity of labelling was different between the homologues and the larger site corresponded to a larger secondary constriction. This labelling pattern was observed consistently in all genotypes. On the other hand, differences in the number of 5S sites were observed between the accessions. While in some of the wild seeded species, the 5S rDNA was localised on two pairs of chromosomes, hybridisation signals appeared on three pairs of chromosomes in other wild accessions. Quite unexpectedly, only five sites of 5S rDNA were reproducibly observed in the two vegetatively propagated diploid edible cultivars, Pisang Mas and Niyarma Yik, evidence for structural heterozygosity. A dual colour FISH showed that in all accessions, the satellite chromosomes carrying the 18S-25S loci did not carry

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the 5S loci. The results demonstrate that molecular cytogenetics can be applied to *Musa* and that physical cytogenetic maps can be generated.

Additional key words: *Musa acuminata*, *Musa balbisiana*, fluorescent *in situ* hybridisation, FISH.

Introduction

Bananas are important staple and cash crops for millions of people in the tropics and subtropics. They are perennial giant herbs of the *Musa* genus. Cheesman (1947) devided the genus into four sections: *Eumusa* (n=11), *Rhodochlamys* (n=11), *Callimusa* (mostly n=10), and *Australimusa* (n=10). Most cultivated bananas belong to the section *Eumusa*. With some exceptions, edible banana cultivars are either seed sterile or exhibit a very low seed set and are vegetatively propagated. It is believed that they originated as a result of natural inter- and intraspecific hybridisation of the diploid *Musa* species, *M. acuminata* Colla. (A genome) and *M. balbisiana* Colla. (B genome) (Simmonds 1962). However, until now none of the triploid or tetraploid cultivars has been traced unambiguously to their wild diploid progenitor species.

The production of bananas is threatened by many diseases caused by pathogenic fungi, bacteria or viruses (Gowen 1995, Robinson 1996). To obtain resistant triploid clones, the resynthesis of triploids from resistant wild diploid species and diploid cultivars was proposed already by Stover and Buddenhagen (1986). However, the weakness of this strategy lies in the fact that the origin of cultivated diploid cultivars is not very clear. While the genetic variation within *M. balbisiana* seems to be small, *M. acuminata* has diversified into several sub-species and many parthenocarpic cultivars.

Several authors used molecular markers to analyse the evolution of *Musa* (Gawel *et al.* 1992, Howell *et al.* 1993, Lanaud *et al.* 1992, Kaemmer *et al.* 1997). Based on the analysis of nuclear, chloroplast and mitochondrial genomes, Carreel (1994) established links between several cultivars and *Musa* species and sub-species, and suggested the involvement of *Musa schizocarpa* in the origin of most cultivated clones. In contrast to the progress in the analysis of the nuclear genome at the molecular level, the knowledge of the genome at the chromosomal level remains poor. Analysis of *Musa* chromosomes has been complicated by difficulties with the preparation of chromosome spreads and the small size (1 to 2 μm) of *Musa* chromosomes (Doležel *et al.* 1998). The size of nuclear genome in *Musa* is only about 600 Mbp (Doležel *et al.* 1994), thus an average chromosome has only a size of about 50 Mbp DNA. This has impeded both the development of a reliable karyotype of *Musa* and the development of a physical cytogenetic map. The map would enable the correlation of molecular markers to specific chromosomes and their regions and integration of physical maps and genetic linkage maps.

Some results obtained recently indicate that high resolution chromosome studies are possible even in *Musa* (Doležel *et al.* 1998) and that molecular cytogenetics may be used to physically localise DNA sequences on *Musa* chromosomes (Doležel 1996, Dolčelová *et al.* 1997). Furthermore, genomic *in situ* hybridisation (GISH) was

successfuly used to determine genomic constitutions of some *Musa* lines (Osuji *et al.* 1997). Ribosomal RNA genes are organised in tandemly repeated units ranging from 250 to 22 000 copies per genome in plants (Rogers and Bendich 1987). Because of potentially large number of loci and large sequence variation both within and between loci, rDNA genes are difficult to map. On the other hand, rDNA clusters can be easily visualised by FISH (Leitch and Heslop-Harrison 1992, Lubaretz *et al.* 1996, Ma *et al.* 1997) which makes them suitable targets for initial studies on physical genome mapping. The aim of this study was to analyse the number and distribution of 18S-25S and 5S rDNA sites on chromosomes of eight diploid *Musa* species and clones.

Materials and methods

Plants and root-tip pretreatment: All diploid (2n=2x=22) *Musa* species and clones used in this study were obtained from the INIBAP Transit Centre (Katholieke Universiteit Leuven, Belgium) as *in vitro* rooted plantlets. After transfer to soil, plants were maintained in a greenhouse. Actively growing roots were cut about 1 cm from the root tip and collected in 50 mM phosphate buffer (pH 7.0) containing 0.2 % β-mercaptoethanol. After a pre-treatment in 0.05 % 8-hydroxyquinoline for 3 h at room temperature, the roots were fixed in ethanol:acetic acid (3:1) fixative and stored in 70 % ethanol.

Metaphase preparation: Chromosome spreads were prepared according to Doležel *et al.* (1998). Briefly, fixed roots were washed in a solution of 75 mM KCl and 7.5 mM EDTA (pH 4). Meristem tips of five to fifteen roots were digested for 60 min at 30 °C in 0.4 cm^3 of enzyme mixture (1 % pectinase, 0.5 % pectolyase, and 0.5 % cellulase) made in 0.1 M citrate buffer (pH 4.7). The suspension of released protoplasts was filtered through a 150 μm nylon mesh and pelleted at 600 *g*. The pellet was resuspended in 0.4 cm^3 of 75 mM KCl and 7.5 mM EDTA (pH 4) and incubated for 5 min at room temperature. After pelleting, the protoplasts were washed three times with 70 % ethanol. Protoplast suspension (0.007 cm^3) was dropped onto a clean ice-cold microscope slide. The suspension was allowed to spread out and air-dry. This process was monitored under a microscope. Shortly before complete drying out, 0.007 cm^3 of ice-cold ethanol:acetic acid (3:1) fixative was added to the drop to induce cell bursting and then rinsed in 100 % ethanol and air-dried at room temperature.

DNA probes: The plasmid VER 17 (Yakura and Tanifuji 1983) containing 5.8S, parts of the 18S and most of 25S genes of *Vicia faba* was cloned into XL1 Blue *E. coli*. To prepare a probe, it was labelled with a Random Primed DNA Labelling Kit (*Boehringer*, Mannheim) according to the manufacturer's instructions. The probe for 5S rDNA was prepared by PCR using a pair of specific primers (RICRGAC1, GATCCCATCAGAACTCCGAAG; RICRGAC2, CGGTGCTTTAGTGCTGGTATG) which amplify 303 bp in rice, and some other plants (Fukui and Ohmido, personal communication). The probes were labelled either with biotin or digoxigenin.

***In situ* hybridisation:** Slide-bound chromosomal DNA was denatured in a solution of 70 % formamide in 2xSSC for 2 min at 72 °C, and equal amount of 0.02 cm^3 of hybridisation mixture was applied to each slide preparation. The hybridization mixture contained 50 % formamide, 20 % dextran sulfate in 2xSSC, 125 mg dm^{-3} sheared calf thymus DNA, and 1 mg dm^{-3} labelled probe. The mixture was denatured at 76 °C for 15 min before application to the slides. The hybridisation was carried out at 37 °C overnight. After removing the cover slips, slides were washed with 50 % formamide in 2xSSC at 37 °C for 10 min, and three times in 2xSSC at room temperature.

The sites of digoxigenin-labelled probe hybridisation were detected using anti-digoxigenin-FITC and the signal was amplified using anti-sheep-FITC. Sites of biotin-labelled probe hybridisation were detected using avidin-Cy3 and the signal was amplified using biotinylated anti-avidin and avidin-Cy3. Alternatively, probe hybridisation were detected using avidin-FITC and the signal was amplified using biotinylated anti-avidin and avidin-FITC.

Fluorescence microscopy: The slides were examined with an *Olympus BX 60* epifluorescence microscope (Prague, Czech Republic) and the images of DAPI, FITC and Cy3 fluorescence were acquired separately with a black and white CCD camera (*PCO CCD Imaging*, Kelheim, Germany). The camera was interfaced to a PC running the ISIS software (*Metasystems*, Belmont, USA). The images corresponding to DAPI, FITC and Cy3 were superimposed after contrast and background optimisation.

Results

Metaphase chromosomes in *Musa* are very small and of similar size. In chromosome spreads, the positions of centromeres could not be unequivocally identified on all chromosomes within a karyotype. The similarity in chromosome size and difficulty to determine centromere position meant that we were not able to identify homologues after Giemsa or DAPI staining. Nevertheless, secondary constrictions could be clearly identified on short arm of a pair of chromosomes both in the AA and BB genotypes (Fig. 1).

In situ hybridisation with the VER17 probe for 18S-25S rRNA genes revealed two sites of hybridisation in all AA and BB genotypes (Table 1). Always the hybridisation sites occurred at the distal end of the short chromosome arm and coincided with a secondary constriction. Interestingly, the intensity of labelling was different for each of the homologue with one of them showing a very intensive labelling. This phenomenon was observed consistently in all genotypes (Fig. 2*A,B*). No minor 18S-25S loci were detected even under the conditions of reduced stringency.

FISH with a probe for 5S rRNA genes resulted in clear and reproducible signals. Although some of them were of low intensity, they were consistently observed in all metaphase plates. No variation in the number of 5S sites within genotypes was

observed. On the other hand, variation in the number of 5S sites was observed between the genotypes (Fig. 2*C,D*). In AA genotypes, the number of 5S sites ranged from four to six (Table 1). In BB genotypes, six sites of labelling with the 5S probe were observed. The only exception being *M. balbisiana* type Cameroun where only four sites were found (Table 1).

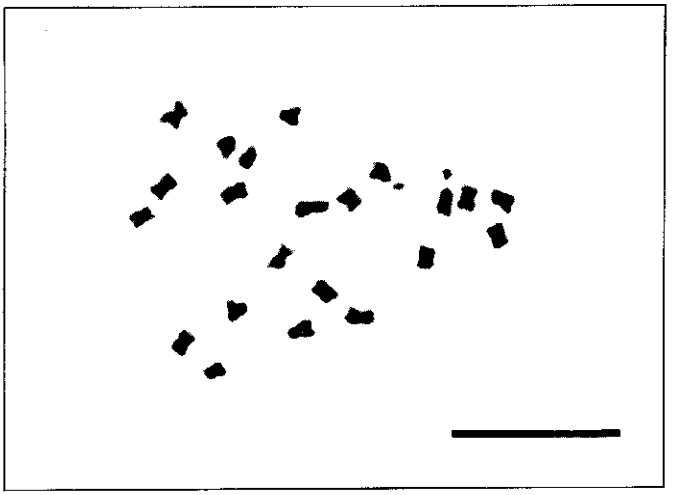

Fig. 1. Mitotic metaphase plates of diploid (2n=22) *Musa acuminata* ssp. *banksii* cv. Niyarma Yik (ITC 0269). *Bar* = 10 μm.

Table 1. The number of 18S-25S rDNA and 5S rDNA sites observed on mitotic chromosomes of diploid (2n=22) *Musa* clones and species. ITC code assigned by the INIBAP Transit Centre (Leuven)

Genotype	Accession	ITC Code	18S-25S rDNA	5S rDNA
M. acuminata ssp. banksii	Higa	0428	2	4
M. acuminata ssp. siamea	Pa (Rayong)	0672	2	6
AA cultivar	Pisang Mas	0653	2	5
AA cultivar	Niyarma Yik	0269	2	5
M. balbisiana	M. balbisiana	0094	2	6
M. balbisiana	Cameroun	0246	2	4
M. balbisiana	Singapuri	0248	2	6
M. balbisiana	Tani	1120	2	6

With the aim to understand the distribution of 18S-25S and 5S rDNA sequences within *Musa* karyotypes, we have performed a two-colour FISH with probes for both sequences. The data confirmed the results obtained with single probes. Furthermore, we have found that both in AA and BB genotypes, the 5S sites are localised on chromosomes which do not carry 18S-25S rRNA genes (Fig. 3).

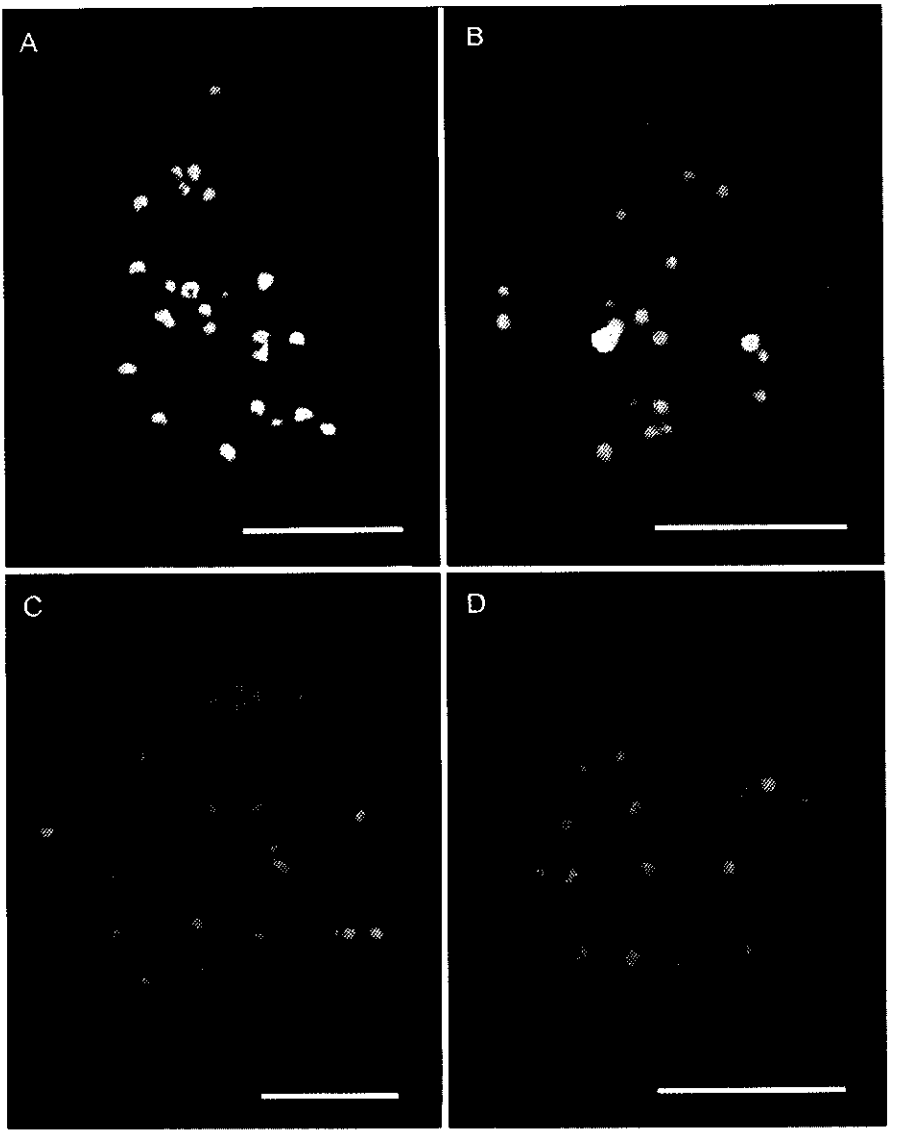

Fig. 2. Mitotic metaphase plates of diploid (2n=22) *Musa* clones and species after *in situ* hybridisation with rDNA probes. The chromosomes were counterstained with DAPI (*blue colour*) and the probes were detected either with FITC (*yellow-green signals*) or Cy3 (*red signals*). *A* - *Musa acuminata* ssp. *banksii* type Higa (ITC 0428) hybridised with a probe for 18S-25S rDNA. The two signals correspond to nucleolar organiser regions. *B* - *M. balbisiana* type Cameroun (ITC 0246) hybridised with a probe for 18S-25S rDNA. The two signals correspond to nucleolar organiser regions. Note the difference in signal intensity between both sites. *C* - Metaphase plate of cv. Pisang Mas (ITC 0653) showing five sites of 5S rDNA. *D* - Metaphase plate of *Musa balbisiana* (ITC 0094) showing six sites of 5S rDNA. *Bar* = 10 μm.

Discussion

In this work, we have determined for the first time the number and distribution of rDNA loci on chromosomes of eight diploid *Musa* species and clones. The 18S-25S rDNA cluster was localised exclusively in the secondary constriction of one chromosome pair in all *Musa* genotypes analysed in this study. The presence of only one chromosome pair with secondary constriction in wild bananas with the AA genome was reported also by Wang *et al.* (1993). In contrast to our results, these

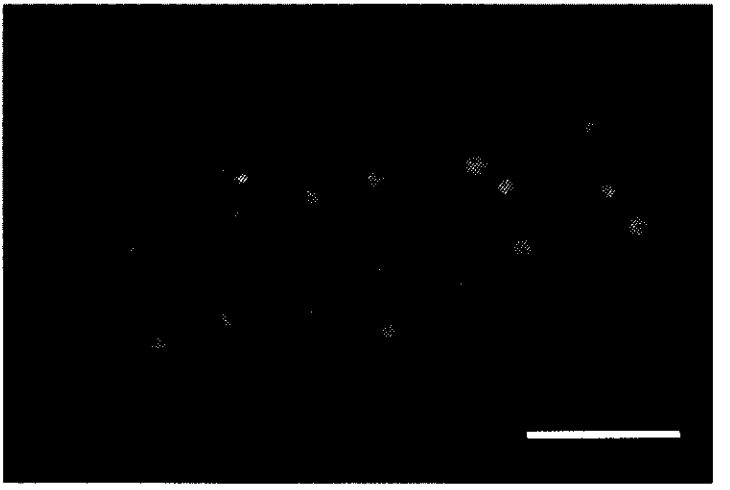

Fig. 3. Mitotic metaphase plate of diploid (2n=22) *Musa acuminata* ssp. *banksii*, type Higa (ITC 0428) after simultaneous *in situ* hybridisation with probes for 18S-25S rDNA (2 sites) and for 5S rDNA (4 sites). The chromosomes were counterstained with DAPI (*blue colour*) and the probes or 18S-25S rDNA and 5S rDNA were detected with FITC (*yellow-green signals*) and Cy3 (*red signals*), respectively. Note that the 18S-25S and 5S rDNA loci are localised on different chromosomes. *Bar* = 10 μm.

authors observed two pairs of satellite chromosomes in wild BB bananas. Although *in situ* hybridisation is not a fully quantitative method (Appels *et al.* 1980), a difference in the intensity of labelling between the homologues probably indicates a difference in the copy number of 18S-25S rRNA genes. In our case this interpretation seems to be supported by the signal intensity being correlated with the size of the NOR region (Fig. 1). The difference in the number of rDNA repeats could be a result of their amplification or reduction. For instance, it is known that tandem repeats can be amplified or deleted in the course of unequal crossing over (Arnheim *et al.* 1980).

While each of eight diploid accessions analysed had only one pair of chromosomes bearing the 18S-25S site, variation in the number of 5S sites (range 4 - 6) was observed. This result indicates intraspecific variation in chromosome structure in *Musa*. The polymorphism in the number of 5S sites is in line with previous studies indicating a large genetic variation within *M. acuminata*. Surprisingly, we have

detected the polymorphism also within *M. balbisiana,* a species which has been considered genetically more homogenous. This observation supports the results of Lanaud *et al.* (1992) who analysed rDNA spacer length in *Musa.* The authors detected variation even at a subspecies level not only within the *acuminata* complex but also within the BB group.

At present we do not have enough data to explain the intraspecific variation in the number of 5S rDNA loci in *Musa.* It is known that the number of multigene loci such as rDNA may be reduced during the evolution of polyploids (Dvořák *et al.* 1990). For instance, the number of additional 5S rDNA sites was reduced during the evolution of the *Brachyscome lineariloba* complex (Adachi *et al.* 1997). The evolution of the amphidiploid *Brassica napus* from the two diploid progenitors (*B. rapa* and B. *oleracea*) was accompanied by a loss of one rDNA locus (Snowdon *et al.* 1997). A difference in the number of rDNA loci at a diploid level was observed between two subspecies of *Oryza sativa* (Fukui *et al.* 1994).

The presence of an odd number of 5S sites in two edible cultivars (Pisang Mas and Niyarma Yik) is a very interesting outcome of this study and is on line with the assumption that structural chromosome heterozygosity occurs in all the diploid edible *Musa* cultivars (Wilson 1946, Hutchinson 1966, Faure *et al.* 1993). While the possibility of chromosome structural changes after reproductive isolation cannot be excluded, an attractive explanation would be that these cultivars originate from hybridisation between two genotypes bearing four and six 5S rDNA sites, respectively.

This study represents an attempt of physical mapping of the *Musa* genome through FISH with rDNA sequences. The technique allowed a more detailed analysis of *Musa* karyotype and provided the first physical landmarks. Future work should concentrate on comparative mapping of other repetitive DNA sequences and large DNA fragments. It may be expected that the study of *Musa* karyotype at the molecular level will have significant impact on the understanding of chromosome structure and evolution of this genus.

References

Adachi, J., Watanabe, K., Fukui, K., Ohmido, N., Kosuge, K.: Chromosomal location and reorganization of the 45S and 5S rDNA in the *Brachyscome lineriloba* complex. - J. Plant Res. **110**: 371-377, 1997.

Appels, R., Gerlach, W.L., Dennis, E.S., Swift, H., Peacock, W.J.: Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. - Chromosoma **78**: 293-311, 1980.

Arnheim, N., Krystal, M., Schmickel, R., Wilson, G., Ryder, O., Zimmer, E.: Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and ape. - Proc. nat. Acad. Sci. USA **77**: 7323-7327, 1980.

Carreel, F.: Etude de la diversité génétique des bananiers (genre *Musa*) à l'aide des marqueurs RFLP. - Thesis. Institut National Agronomique Paris-Grignon, Paris 1994.

Cheesman, E.E.: The classification of the bananas. - Kew Bull. **2**: 97-117, 1947.

Doležel, J.: Application of karyology and cytometry in mutation breeding of African plantain *in vitro*. - In: Report of the First FAO/IAEA Research Co-ordination Meeting on Cellular Biology

and Biotechnology Including Mutation Techniques for Creation of New Useful Banana Genotypes. Pp. 13-22. IAEA, Vienna 1996.

Doležel, J., Doleželová, M., Novák, F.J.: Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*). - Biol. Plant. **36**: 351-357, 1994.

Doležel, J., Doleželová, M., Roux, N., Van den Houwe, I.: A novel method to prepare slides for high resolution chromosome studies in *Musa* spp. - Infomusa 1998 (in press).

Doleželová, M., Valárik, M., Doležel, J.: Molecular cytogenetics of *Musa* spp. - In: Abstracts of the Spring Symposium on Plant Cytogenetics. P. 22. Silesian University, Cieszyn 1997.

Dvořák, J.: Evolution of multigene families: The ribosomal RNA loci of wheat and related species. - In: Brown, A.H.D. (ed.): Plant Population Genetics, Breeding and Genetic Resources. Pp. 83-97. Sinauer Associate Inc. Publ., Sunderland 1990.

Faure, S., Bakry, F., Gonzales de Leon, D.: Cytogenetic studies of diploid bananas. - In: Breeding Banana and Plantain for Resistance to Disease and Pests. Pp. 77-92. CIRAD/INIBAP, Montpellier 1992.

Fukui, K., Ohmido, N., Khush, G.S.: Variability in rDNA loci in genus *Oryza* detected through fluorescent *in situ* hybridization. - Theor. appl. Genet. **87**: 893-899, 1994.

Gawel, N.J., Jarret, R.L., Whittemore, A.P.: Restriction fragment length polymorphism (RFLP)-based phylogenetic analysis of *Musa*. - Theor. appl. Genet. **84**: 286-290, 1992.

Gowen, S.: Bananas and Plantains. - Chapman and Hall, London 1995.

Howell, E.C., Newbury, H.J., Swennen, R.L., Withers, L.A., Ford-Lloyd, B.V.: The use of RAPD for identifying and classifying *Musa* germplasm. - Genome **37**: 328-332, 1993.

Hutchinson, D.J.: Translocation configurations in a diploid banana. - Can. J. Genet. Cytol. **8**: 184-187, 1966.

Kaemmer, D., Fischer, D., Jarret, R.L., Baurens, F.-C., Grapin, A., Dambier, D., Noyer, J-L., Lanaud, C., Kahl, G., Lagoda, P.J.L.: Molecular breeding in the genus *Musa*: a strong case for STMS marker technology. - Euphytica **96**: 49-63, 1997.

Lanaud, C., Tezenas du Montcel, H., Jolivot, M.P., Glaszmann, J.C., De Leon, D.G.: Variation of ribosomal gene spacer length among wild and cultivated banana. - Heredity **68**: 147-156, 1992.

Leitch, I.J., Heslop-Harrison, J.S.: Physical mapping of the 18S-5.8S-26S rRNA genes in barley by in situ hybridisation. - Genome **35**: 1013-1018, 1992.

Lubaretz, O., Fuchs, J., Ahne, R., Meister, A., Schubert, I.: Karyotyping of three *Pinaceae* species *via* fluorescent *in situ* hybridization and computer-aided chromosome analysis. - Theor. appl. Genet. **92**: 411-416, 1996.

Ma, Y., Islam-Faridi, M.N., Crane, C.F., Ji, Y., Stelly, D.M., Price, H.J., Byrne, D.H.: *In situ* hybridization of ribosomal DNA to rose chromosomes. - J. Hered. **88**: 158-161, 1997.

Osuji, J.O., Harrison, G., Crouch, J., Heslop-Harrison, J.S.: Identification of the genomic constitution of *Musa* L. lines (bananas, plantains and hybrids) using molecular cytogenetics. - Ann. Bot. **80**: 787-793, 1997.

Robinson, J.C.: Bananas and Plantains. - CAB International, Wallingford 1996.

Rogers, S.O., Bendich, A.J.: Ribosomal RNA genes in plants: A variability in copy number and in the intergenic spacer. - Plant mol. Biol. **9**: 509-520, 1987.

Simmonds, N.W.: Evolution of the Bananas. - Longman, London 1962.

Snowdon, R.J., Kohler, W., Kohler, A.: Chromosomal localization and characterization of rDNA loci in the *Brassica* A and C genomes. - Genome **40**: 582-587, 1997.

Stover, R.H., Buddenhagen, I.W.: Banana breeding, polyploidy, disease resistance and productivity. - Fruits **41**: 175-191, 1986.

Wang, Z., Lin, Z., Pan, K.: Cytogenetical studies in *Musa* (*Eumusa*). - In: Current Banana Research and Development in China. Pp. 29 - 43. South China Agricultural University, Guangzhou 1993.

Wilson, G.B.: Cytological studies in the *Musae*. II. Meiosis in some diploid clones. - Genetics **31**: 475-482, 1946.

Yakura, K., Tanifuji, S.: Molecular cloning and restriction analysis of *Eco*RI-fragments of *Vicia faba* rDNA. - Plant Cell Physiol. **24**: 1327-1330, 1983.