Revision on banana taxonomy: 'Klue Tiparot' (*Musa* sp) reclassified as a triploid

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Revision on banana taxonomy: 'Klue Tiparot' (*Musa* sp) reclassified within triploids.

ABSTRACT

INTRODUCTION. 'Klue Tiparot' is a well-known international reference, described as a natural tetraploid banana. It can be easily recognised by its natural tendency for the male axis to be hidden under the bunch. 'Klue Tiparot', present in the reference collections of Cirad-Flhor, Crbp and Inibap, originated from a collecting mission in Thailand. Its particular characteristics and its function as a reference clone lead the authors to give a complete description including morphological, cytological and molecular aspects. **MATERIALS AND METHODS.** The plant was described from observations of the collections in Guadeloupe and Cameroon. In order to determine its ploidy level, flow cytometry and chromosome counting was carried out. Three control clones were then selected: a diploid wild AA clone, a triploid typical ABB and a confirmed tetraploid. Molecular analysis of the cytoplasmic and nuclear genome were conducted using the RFLP technique. **RESULTS.** the 'Klue Tiparot' agromorphological description was in agreement with previous characterization. The ploidy level determination lead to the conclusion that it is a triploid clone and not a tetraploid one. Integration of the morphotaxonomic data with the molecular analysis of nuclear and cytoplasmic genomes led us to reclassify 'Klue Tiparot' in the ABB group. **DISCUSSION** These new results differ from previous results. It is argued that the present botanical classification of 'Klue Tiparot' will have to be modified. **CONCLUSION.** The clone, present in the main field genebanks is, in fact, a triploid clone.

KEYWORDS
Musa, classification, chromosome number.

Révision taxinomique : « Klue Tiparo » (*Musa sp*) reclassé parmi les bananiers triploïdes.

RÉSUMÉ

INTRODUCTION. « Klue Tiparot » est une référence internationale, connue comme un bananier tétraploïde naturel. Il est facilement identifié par sa tendance à présenter son axe mâle caché sous le régime. Le clone, présent dans les collections de référence du Cirad-Flhor, du CRBP et de l'Inibap, provient d'une prospection effectuée en Thaïlande. Ses caractéristiques et son intérêt global en tant que clone de référence ont conduit les auteurs à le décrire précisément à partir de ses aspects morphologiques, cytologiques et moléculaires. **MATÉRIEL AND MÉTHODES.** Les plants de collections disponibles en Guadeloupe et au Cameroun ont été décrits. Les techniques de cytométrie en flux et de comptage des chromosomes ont été utilisées pour déterminer le niveau de ploïdie. Pour cela, trois clones de références ont été choisis : un diploïde sauvage AA, un triploïde typique ABB et un tétraploïde confirmé. Les génomes cytoplasmique et nucléaire ont été analysés par la technique des RFLP. **RÉSULTATS.** La description agromorphologique du « Klue Tiparot » est conforme à sa caractérisation classique. La détermination du niveau de ploïdie a abouti à la détermination d'un triploïde et non d'un tétraploïde. La confrontation des données morphotaxinomiques et des analyses moléculaires des génomes nucléaire et cytoplasmique conduit à reclasser « Klue Tiparot » dans le groupe AAB. **DISCUSSION.** Ces nouveaux résultats sont originaux. L'argumentation avancée conduirait à modifier l'actuelle classification botanique du « Klue Tiparot ». **CONCLUSION.** Ce clone, présent dans les principales banques de gènes du bananier est, en fait, un triploïde.

MOTS CLÉS
Musa, classification, nombre chromosomique.

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introduction

The family Musaceae belongs to the order Zingiberales and contains two genera: *Musa* and *Ensete*. The genus *Musa* is divided into four sections: *Australimusa, Callimusa, Rhodochlamys* and *Eumusa*. *Eumusa* was the source of the great majority of the cultivated bananas. These edible bananas are mostly derived from *Musa acuminata* and *Musa balbisiana* (SIMMONDS, 1962), two wild species belonging to the section *Eumusa*. Recently, it has been confirmed that two other species contributed to the creation of some cultivated clones, at least in a minor way: *Musa schizocarpa* and a species from the *Australimusa* section (CARREEL, 1994).

'Klue Tiparot', a well-known international reference, is a banana clone native to Indochina. SIMMONDS and SHEPHERD (1955) describe it as the only natural tetraploid banana. It is a vigorous plant with massive fruits, truncated and with a grey-green colour. As STOVER and SIMMONDS (1987) stated, it can be easily recognised by its natural tendency for the male axis to be hidden under the bunch. When this male axis is present, it is normally developed, otherwise it is completely hidden behind the last female hand. Furthermore, one of its Thai names, 'Klue Plihai' – banana with hidden flowers – refers to this behaviour. In several cases, this clone bears two names according to the kind of inflorescence ('Pisang Abu Siam' when the male axis is present, and 'Pisang Abu Nipah' when the male axis is absent, in Malaysia). A lot of names given to this clone (eg, 'Pisang Batu') in Malaysia refer to *Musa balbisiana* to which it is obviously very close morphologically.

'Klue Tiparot' is present in the collections of Cirad-Flhor[1] (Guadeloupe) and CRBP[2] (Cameroon). These plants were introduced to both countries in 1986 as in vitro plantlets under the code THA 020: they originated from a collecting mission in Thailand by Tézenas du Montcel (Cirad-Irfa[3]), SHEPHERD and FERREIRA (CNPMF[4, 5]). In 1989, Cirad-Flhor provided 'Klue Tiparot' to the International Network for the Improvement of Bananas and Plantains (Inibap) reference collection.

Both these particular characteristics and its function as a reference clone lead us to give a complete description of 'Klue Tiparot', including morphological, cytological and molecular aspects. All these data are gathered here, and a new subsequent classification is proposed.

materials and methods

agromorphological description

The plant has been morphologically described using the MUSAID software, developed by Cirad-Flhor; it is an interactive software package using a database containing 123 characters to evaluate an unidentified clone (PERRIER and TÉZENAS DU MONTCEL, 1990).

reference clones for flow cytometry

Three control clones were used in this study.

At the diploid level, the wild *Musa acuminata banksii* 'Pahang' was chosen. Chromosome counting using classical microscopy confirmed that this clone is exactly diploid (2n = 2x = 22).

'Monthan', also known under the Indian name of 'Pacha Bontha Bathees', was chosen as the triploid reference, mainly because, according to RICHARDSON et al (1965), "*from a comparison of the morphological features of the tetraploid with ABB triploids, it would appear that some member of the 'Pacha Bontha Bathees' alliance is likely to be the maternal ancestor*". 'Monthan' is a typical ABB cultivar, with large angular fruits.

'Yawa 2' has been used as a tetraploid reference. It is a very massive and vigorous cultivar, which was collected by SHARROCK and TÉZENAS DU MONTCEL at Ulautava, Rabaul region in Papua New Guinea in March 1988. Collection notes classify it as a putative tetraploid ABBB. However, some morphotaxonomic criteria, such as the

[1] Cirad-Flhor is the Fruit and Horticultural Department of the Centre de coopération internationale en recherche agronomique pour le développement (Montpellier, France)

[2] Centre régional de recherches bananiers et plantains (Cameroon)

[3] Cirad-Irfa is the previous name of Cirad-Flhor (before 1993)

[4] Centro Nacional de Pesquisa de Mandioca e Fruticultura (Brazil)

[5] 'Klue Tiparot', originating from this collecting mission, has also been provided to Brazil under the accession code THA 020 in April 1987

shape and aspect of the bracts of the male bud, suggest that it might not be a pure ABBB. Indeed CARREEL (1994), using RFLP study of the nucleic and cytoplasmic genomes, confirmed an observation from SHEPHERD (personal communication) and showed the presence of the *Australimusa* genome in 'Yawa 2'. Consequently, the most probable genomic composition of this clone should be ABBT and not ABBB. The letter T is the common abbreviation for the genome of *Musa textilis*, wild form of *Australimusa*. It is used here as a reference to the section *Australimusa* and not specifically for *Musa textilis*. Regardless, this result does not call into question its 4x ploidy level, verified using flow cytometry.

protocol used for the analysis in flow cytometry

Flow cytometry is a rapid and robust technique that allows accurate determination of DNA content in a large number of nuclei. This technique quantifies the intensity of light emitted by isolated nuclei stained by DNA specific fluorochromes once excited.

• Isolation and staining nuclei: according to DOLEZEL et al (1989), approximately 50 mg of young leaves, taken off plants in field collection, were chopped with a razor blade in a 55 mm diameter plastic petri dish containing 2 ml LB01 fresh lysis buffer. The suspension of released nuclei was passed through a 15 µm nylon filter and supplemented with 15 µg of DAPI which is an A-T base pair binding fluorescent dye. Preparations were maintained 5 min in the dark, on ice before analysis. Samples to be analysed were always chopped together and mixed with the control 'Pahang'.

• Flow cytometry: measures were performed on a Bryte HS flow cytometer using UV excitation wavelengths provided by a 75 W Xenon-Mercury lamp. Histograms of intensity were registered over 256 channels and studied on a 486 DX 33 Dell PC using WinBryte software Ver 1.0.1. During analysis, the peak of the histogram obtained with the diploid 'Pahang' control was arbitrarily placed on the 50 channel.

chromosome counting using classical microscopy

Chromosome counting was carried out using the method defined by SHEPHERD (personal communication). The root tips were taken from plants between 6.30 am and 8.00 am and placed in 0.03% w/v hydroxyquinoline solution, open to the air at 20–25°C for 6–8 h.

The tips were then immersed for at least 3 h in a macerating/fixing solution (AcOH:EtOH: water; 4:1:5). Finally, the root tips were crushed between a microscope slide and cover-slip in a drop of stain (orcein 2% in lactic acid:phenol:-glycerol:water; 1:1:1:1). Counting was performed at a 1000 × magnification.

molecular marker analysis

Molecular analysis of the cytoplasmic and nuclear genome were conducted using the RFLP technique with heterologous and homologous probes (CARREEL, 1994).

results

agromorphological description

Our observations have shown that the plant in the collection at Neufchâteau (Cirad-Flhor, Guadeloupe) is identical to the plant initially observed in the collection at CRBP. Using the MUSAID program, 'Klue Tiparot' can be described as follows:

• Vegetative stage: robust plant with slightly drooping leaves. Pseudostem very waxy, pure green and dull coloured. Under sheaths are apple green with a slight pink pigmentation. First sucker is often higher than parent. Small brown black blotches develop at the base of the petiole. Petiole canal is closed to overlapping. Petiole margins are green and not winged. No blotches on leaves of young suckers.

• Inflorescence: peduncle is glabrous, dark green coloured, with two or more empty nodes. Position of the bunch depends on its type (see below). When complete, bunch hangs at an angle of 45%, asymmetric and lax. Rachis when present falls ver-

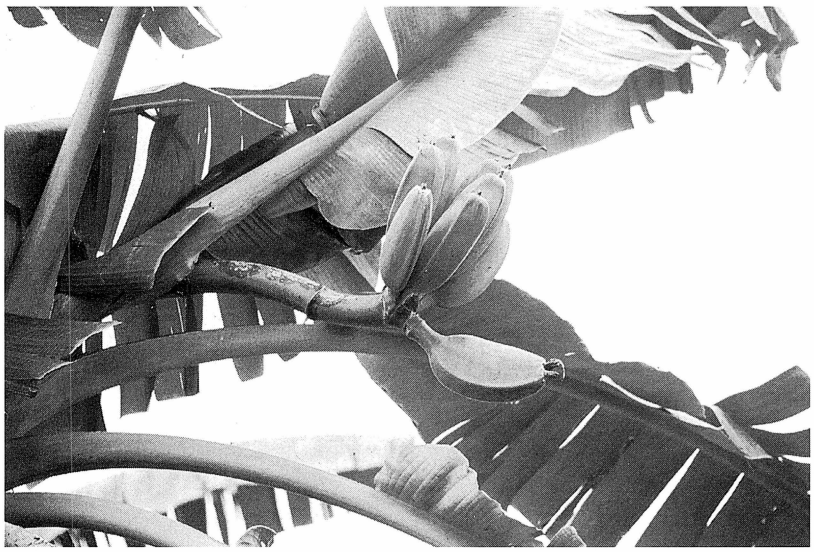

Figure 1
True-horn shape bunch of the 'Klue Tiparot' clone.

tically. Neutral flowers persist under the bunch. Male bud is present.

• Male bud and bracts: base of the bract is slightly shouldered. Apex obtuse and split. Young bracts greatly overhanging. In colour the bract is red, purple outside and very waxy.

• Male bud and male flowers: compound tepal orange with pink pigmentation. Tepal lobes are orange. Free tepal is more or less smooth, with apex triangular and medium development. Anthers are larger than compound sepal. Pollen is absent. Style is white without pigmentation. Ovary is cream with red and purple pigmentation. Dominant colour of male flower is pink.

• Fruit characteristics: fruits are perpendicular to the peduncle. Each hand holds around ten fruits. Fruit size ranges from 15 to 20 cm. Ridges are pronounced and apex is rounded. Style is persistent. Peduncles are large, not hairy and not fused. Skin is thick and grey-green turning bright yellow when ripe.

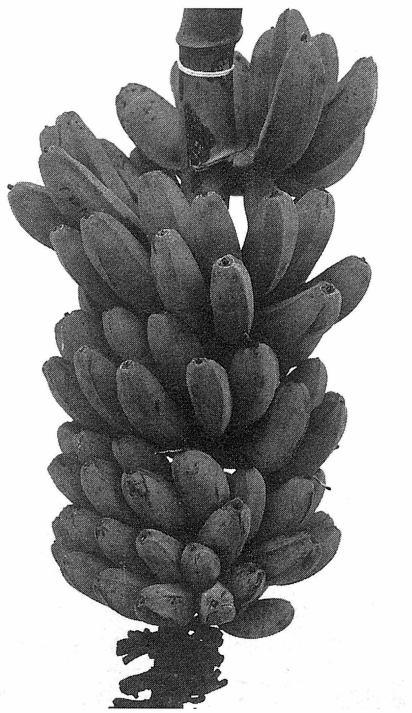

Figure 2
Complete bunch of the 'Klue Tiparot' clone.

All these morphological observations agree with previous descriptions of 'Klue Tiparot' (RICHARDSON et al, 1965; STOVER and SIMMONDS, 1987). Moreover, the main morphological characteristic of 'Klue Tiparot', according to these authors, is its bunch instability. And, in fact, we have observed that it varies between two completely different forms. The characteristic shape of one form resembles a true-horn plantain bunch (fig 1): the bunch is horizontal or slightly erect with one or two hands only, and the rachis is truncated after the last hand. Fruits are large and angular, with a truncated apex. Conversely, the classical form is a pendulous bunch with seven-eight hands and more than 60 fruits, also large and angular; rachis is dressed under the bunch and the male bud is typical of an ABB clone (fig 2).

Both these inflorescence types have been observed in the field and on the same plant in the collection at CRBP. Two bunches were observed in 1995 at Ciradf-Flhor and both were complete. The true-horn shape of the bunch was however observed on these same plants during the preceding cycles, and again in 1996. One bunch also showed a tendency to helicoidal organisation. The bracts of the male bud were coming off entire and in one piece, in a spiral sequence. In fact, the helix was not perfect and numerous distinct scars were present on the rachis. However, some male hands were effectively settled continuously on the scape. All scars were at least very close to each other. This helicoidal structure of the bunch is very rare, and mainly known in 'Pisang

Rojo Uter' (AA cultivar) and 'Ndingo Liko' (AAB plantain).

flow cytometry results

Flow cytometric analysis of plant material yielded high-resolution DNA histograms (figs 3, 4). Coefficients of variation (CV) ranged from 2.3 to 5.6, according to the clones. The first analysis of 'Klue Tiparot' had a double control test, using the diploid reference 'Pahang' and the tetraploid control 'Yawa 2'. Pairwise comparisons of all clones were studied and the last analysis was made with the three clones mixed in the same sample. Results are given in table I.

The different ratios of peak positions reflect the respective ploidy levels. For a 2x/3x situation, resulting ratio is 1.5 ± 0.05, and for a 2x/4x situation, 2.0 + 0.20. Analysis 1 shows that 'Klue Tiparot' may be a triploid clone, when compared to diploid reference 'Pahang' (fig 3, plot 1). Analysis 2 confirms that 'Yawa 2' is really a tetraploid reference (ratio 4:2 with 'Pahang'; fig 3, plot 2). Analysis 3 confirms that the ploidy levels of 'Klue Tiparot' and 'Yawa 2' are different and, finally, analysis 4 shows the definitive hierarchy of ploidy levels of 'Pahang', 'Klue Tiparot' and 'Yawa 2' (respectively 2x, 3x, 4x). 'Klue Tiparot' was intermediate between the diploid clone 'Pahang', and the tetraploid one 'Yawa 2'

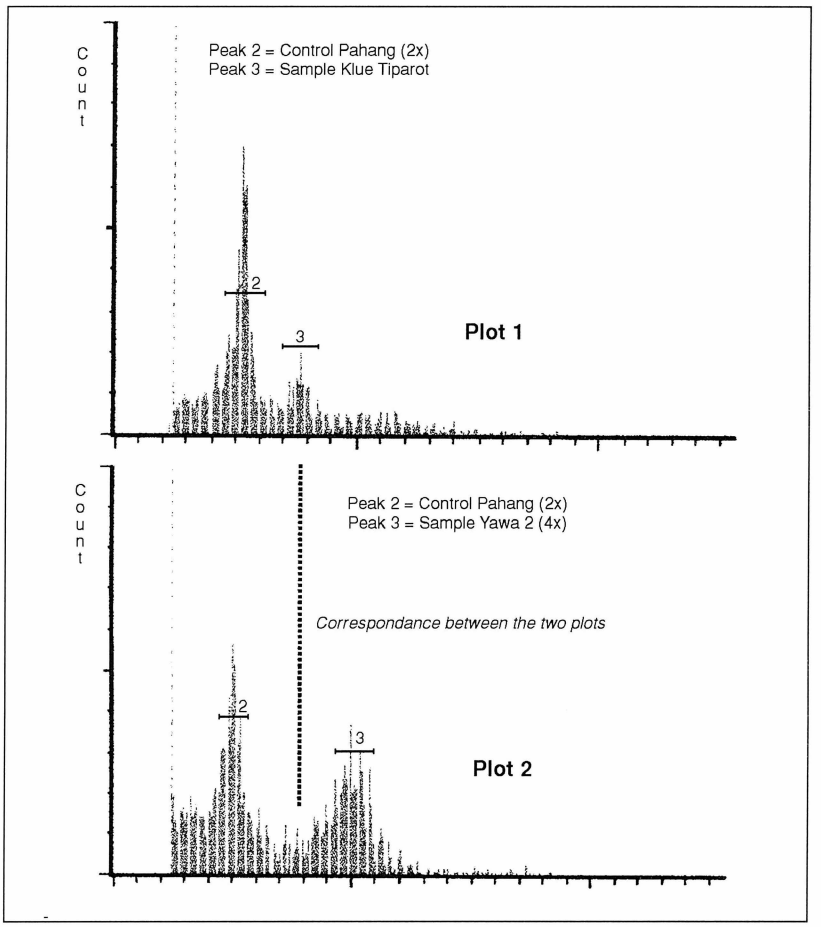

Figure 3
Flow cytometry analysis of he 'Klue Tiparot' and 'Yawa 2' clones.

Table I
Comparative results of the flow cytometry analysis of the clones 'Klue Tiparot' and 'Yawa 2'. Control = *Musa acuminata malaccensis* 'Pahang'.

		Average value of the peak	*Ratio measure: control*
Calibration		50	
Measure 1	Control 'Pahang'	52	
	'Klue Tiparot'	77	1.481
Measure 2	Control 'Pahang'	50	
	'Yawa 2'	99	1.980
Measure 3	'Klue Tiparot'	66	
	'Yawa 2'	90	1.364
Measure 4	Control 'Pahang'	48	
	'Klue Tiparot'	74	1.542
	'Yawa 2'	106	2.208

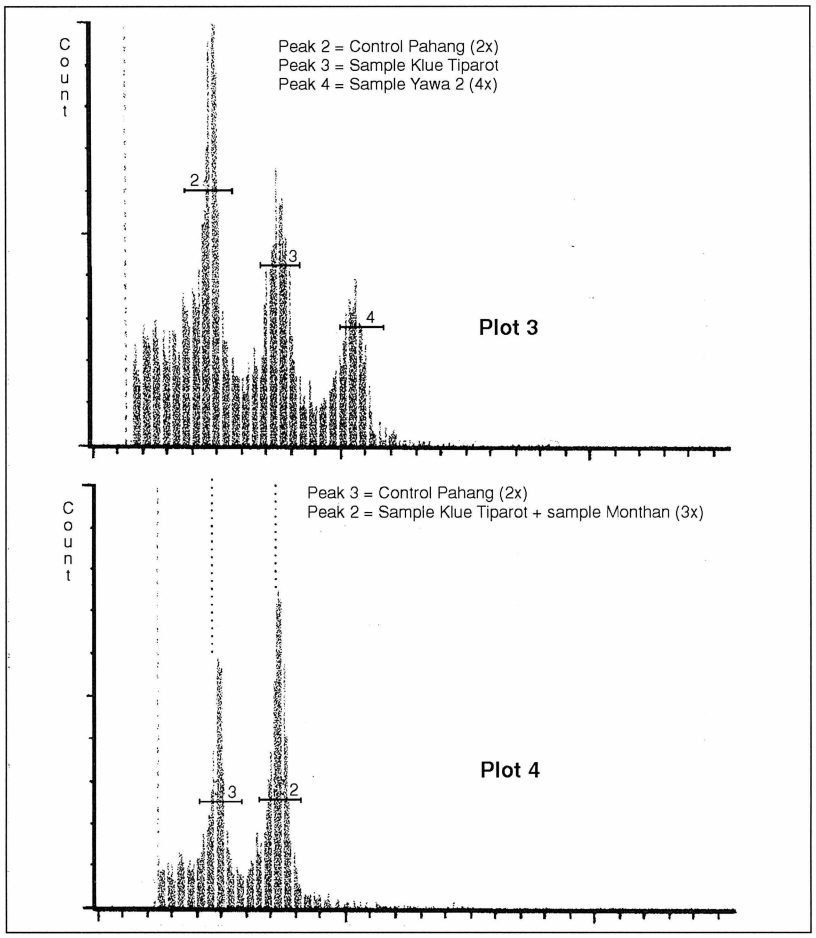

Figure 4
Flow cytometry analysis of the 'Klue Tiparot', 'Yawa 2' and 'Monthan' clones.

(fig 4, plot 3). In order to confirm the supposed 3x ploidy level, samples of 'Klue Tiparot' were mixed together with samples of the ABB control 'Monthan'. Results are given in table II. Analysis 5 confirms that 'Monthan' is a triploid clone. Analysis 6 shows that 'Monthan' and 'Klue Tiparot', analysed together, have the same ploidy level: measures are merged in one peak only, showing a 1.521 ratio with 'Pahang' (2x/3x situation; fig 4, plot 4).

chromosome counts results

Three preparations with 22 distinguishable chromosomes showed 'Pahang' to be definitely diploid (fig 5). In the root tip preparations of 'Klue Tiparot', chromosomes were less clearly defined, making it difficult to ascertain the exact chromosome number (fig 6; table III).

Among the 12 preparations, 10 were of medium quality, indicating a ± one or two chromosomes over 3x ploidy level. Most of the preparations showed a chromosome number ranging from 33 to 36 chromosomes, with a mean of 34 chromosomes. A very good quality preparation revealed 33 chromosomes. No preparation showed less than 33 or more than 36 chromosomes. These results confirm that our accession of 'Klue Tiparot' is not a tetraploid clone, but of triploid constitution with some doubt about the exact chromosome number.

molecular marker analysis results

Analysis of the nuclear genome (CARREEL, 1994) revealed that 'Klue Tiparot' is heterozygous for 44% of the probes studied. Moreover, only three probes out of 42 sho-

Table II
Comparative results of the flow cytometry analysis of the clones 'Klue Tiparot' and 'Monthan'. Control = *Musa acuminata malaccensis* 'Pahang'.

		Average value of the peak	*Ratio measure: control*
Calibration		50	
Measure 5	Control 'Pahang'	50	
	'Monthan'	74	1.480
Measure 6	Control 'Pahang'	48	
	'Klue Tiparot' + 'Monthan' (same peak)	73	1.521

wed three alleles and none shows four alleles. Numerous alleles are identical to those of *Musa balbisiana*, although it also carries specific alleles of *Musa acuminata*. Turning to the cytoplasmic genome, and compared to chloroplastic and mitochondrial patterns of different *Musa* species, 'Klue Tiparot' is closest to *Musa balbisiana*.

As for its relations with cultivated clones, the chloroplastic genome pattern is the same as the ABB clone 'Pisang Kepok', and the closest nuclear patterns are those of the ABB clones 'Pisang Kepok', 'Pelipita' and 'Saba'.

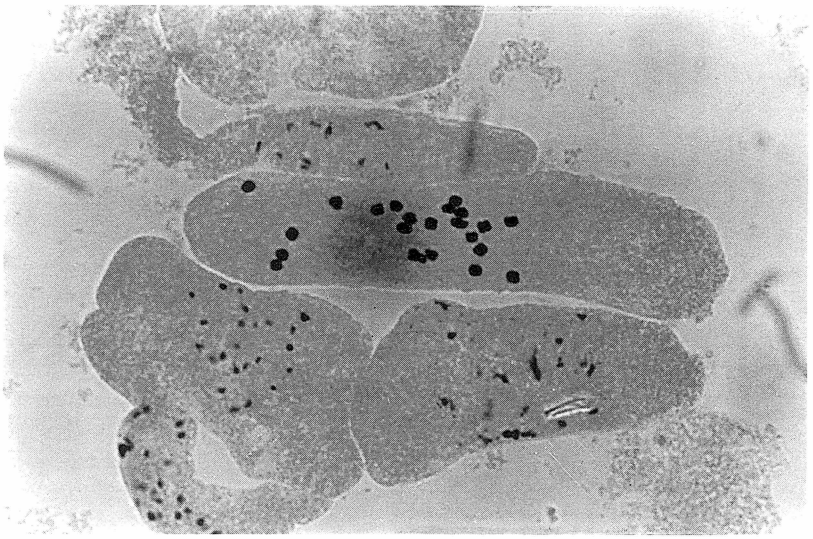

Figure 5
Cytological observation of Musa acuminata malaccensis *type 'Pahang' showing a 22 chromosome set.*

discussion

Counting chromosomes in bananas in order to determine the ploidy of the plants was first used at the beginning of the XXth century. Although maybe "*the initial identification of tetraploid bananas was a report by* TISCHLER *(1910) of the clone 'Pisang Kladi' from Buitenzorg, Java, in which he counted 48 chromosomes*" (RICHARDSON et al, 1965), the first important results arose in the late twenties with WHITE (1928): "*[...] White has also counted the chromosomes in more than a hundred and fifty varieties of* Musa, *using chiefly root tips, and finds an excellent polyploid series, of which he is convinced that the basic haploid number is 4, though he was unable to find a variety having so low a number*" (CHEESMAN, 1932). Starting from this basic number, White found 'Klue Tiparot' to be a decaploid clone with 40 chromosomes, but was not able to carry out the field observation of the plant.

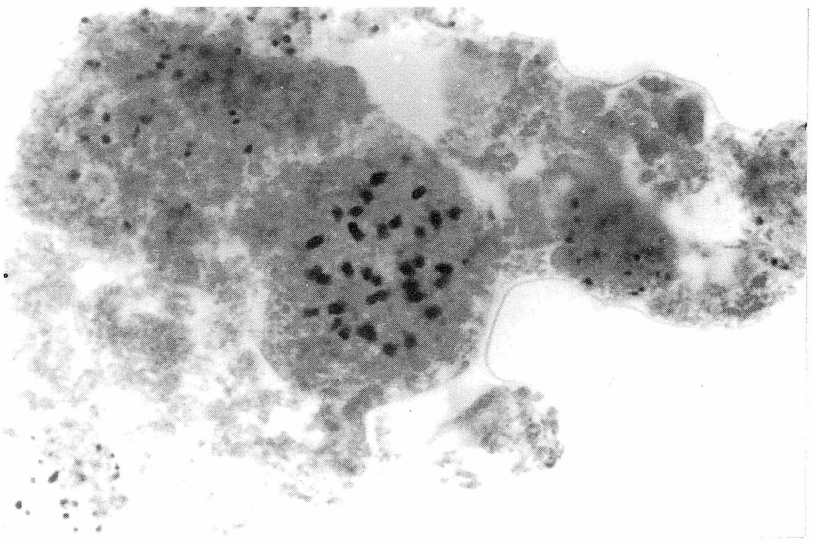

Figure 6
Cytological observation of the 'Klue Tiparot' clone showing a 34 chromosome set.

Table III
Results of the chromosome counts of 'Klue Tiparot'. Quality of the preparation: low level: ± 2-3 chromosomes, medium: ± 1–2 chromosomes, high level: ± 0–1 chromosomes.

Quality of the preparation	*Chromosome number* 33	34	35	36	*Total*
Low level	–	–	–	1	1
Medium	2	5	2	1	10
High level	1	–	–	–	1
Total	**3**	**5**	**2**	**2**	**12**

Four years later, following different results of crosses between Gros Michel and *Musa acuminata malaccensis*, CHEESMAN (1932) disagreed with the White hypothesis and stated that: "*the course of events suggests that the basic haploid chromosome number in this series is eleven*". Given these results, CHEESMAN and LARTER (1935) stated that determination of the true number is of fundamental importance in its bearing on the banana breeding programmes. They analysed 27 fertile *Musa* varieties and 92 seedless edible banana varieties: all of them were diploid (2n=22) or ranging around the triploid level (2n=33, 2n=32 or 2n=34), but no tetraploid was found. Indeed, in 1950, SIMMONDS stated that "*no naturally-occurring tetraploids are known*".

However, SIMMONDS and SHEPHERD (1955), in their fundamental article on *The taxonomy and origins of the cultivated bananas*, revised their judgement. With reference to 'Klue Tiparot', they said that "*only one naturally occurring tetraploid is known*", and that "*it has been shown [...] to be of ABBB type*".

In 1965, RICHARDSON et al carried out the cytological analysis of 648 clones coming from the collections of the United Fruits Company. They found eight tetraploids in this study which they classified in the following way:

– two clones belonging to the group IC-2 (AAAA): the true IC-2 and the 'Banano Honduras', which they believed, in fact, to be synonyms,

– two clones from the Atan group (AAAB): 'Atan' and 'Kudu Kudu',

– two clones from the Kalamagol group (AABB): 'Kalamagol' and 'Unnamed III-186',

– two clones from the Tiparot group (ABBB): 'Balonkawe' and 'Pisang Abu Gagah' which were also supposed to be synonyms of 'Klue Tiparot'.

RICHARDSON et al (1965) analysed the work of WHITE (1928) and wrote that: "*One of these clones, a Philippine variety 'Tiparot', was undoubtedly the clone referred to by SIMMONDS and SHEPHERD (1955)*". They interpreted the 40 chromosome count of White as being too low by four chromosomes compared to their own result.

This work has since been the basis for the proposed ploidy level of 'Klue Tiparot'. Since, that time, 'Klue Tiparot' has been widely used as the natural tetraploid reference in bananas.

Our new results, showing 'Klue Tiparot' to be a triploid clone, differ from these previous results. There are two hypotheses to explain this difference. It could originate in a mistake when choosing our 'Klue Tiparot' samples. But the description of the clone and the characteristics of the description with the specific behaviour of the inflorescence leave little doubt as to the identification of the plant. Moreover, in his collecting mission report, TÉZENAS DU MONTCEL (1985) is certain that it is the classical 'Klue Tiparot', « *bien connu dans ses deux formes (inflorescence complète et sans axe mâle)* ».

The other possibility is then that previous counts were not exact, maybe due to the lack of sufficient technology at that time, to carry out accurate microscopic observation.

It is also true that a typical feature of tetraploid plants in the field is the drooping of the leaves. This morphological characteristic has been widely used as the first criterion of tetraploidy for the description of a plant in the field. But we know now, having observed several synthetic hybrids produced by different breeding programs in the world, that a slight deviation from the exact chromosome number of each ploidy level also induces drooping leaves. We believe that 'Klue Tiparot' is, in fact, a triploid clone, having, possibly, 34 chromosomes.

The fact that molecular analysis revealed only three probes out of 42 with three alleles and none with four alleles is also in agreement with its new ploidy classification.

conclusion

The clone named 'Klue Tiparot', present in the field genebanks of Cirad-Flhor in Guadeloupe, CRBP in Cameroon and the transit centre of Inibap at Leuven (Belgium), seems to be the classic 'Klue Tiparot' known in the literature as the natural tetra-

ploid banana reference and is, in fact, a triploid clone, according to flow cytometry results. Molecular analysis suggests reclassification within the ABB group.

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Revisión taxonómica en el banano: 'Klue Tiparot' (*Musa* sp) es probablemente triploide.

RESUMEN

INTRODUCCIÓN. 'Klue Tiparot' es una referencia internacional conocida como el único banano tetraploide natural, que se identifica fácilmente por su tendencia a tener su eje macho oculto bajo el racimo. El clon presente en las colecciones de referencia del Cirad-Flhor, el CRBP y el Inibap procede de una prospección efectuada en Tailandia. Sus características y su interés global como clon de referencia condujeron a los autores a describirlo precisamente a partir de sus aspectos morfológicos, citológicos y moleculares. **MATERIAL Y MÉTODOS.** Se describieron las plántulas de las colecciones disponibles en Guadalupe y Camerún. Para determinar el nivel de ploidia, se utilizaron las técnicas de citometría en flujo y de recuento de cromosomas. Con tal fin, se eligieron tres clones de referencia: un diploide silvestre AA, un triploide típico ABB y un tetraploide confirmado. Los genomas citoplasmático y nuclear se analizaron mediante la técnica de los RFLP. **RESULTADOS.** La descripción agromorfológica del 'Klue Tiparot' es conforme con su característica clásica. La determinación del nivel de ploidia resultó en la determinación de un triploide y no de un tetraploide. El cotejo de los datos morfotaxonómicos y los análisis moleculares de los genomas nuclear y citoplasmático condujo a volver a clasificar 'Klue Tiparo' en el grupo AAB. **DISCUSIÓN.** Estos nuevos resultados son originales y la argumentación avanzada podría conducir a modificar la actual clasificación botánica del 'Klue Tiparot'. **CONCLUSIÓN.** Este clon, presente en los principales bancos de genes del banano, es en realidad un triploide.

PALABRAS CLAVES

Musa, clasificación, número de cromosomas.