Universities Research Journal 2008
Vol. 1, No. 1

Editorial Board

Prof. Daw Thai Thai Aye, Head of Department of Botany, University of Yadanabon
Prof. Daw Tin Ye, Head of Department of Botany, University of Meiktila
Prof. Nay Win, Head of Department of Botany, University of Kyaukse
Prof. U Aung Myint Thein, Head of Department of Botany, University of Kalay
Prof. Swe Mar Tin, Head of Department of Botany, University of Lashio
Asso. Prof. Dr. San San Aye, Head of Department of Botany, University of Kyainge Tong

Contents

Genetic Diversity and Relationships Among the Myanmar Banana Varieties Using PBA Molecular Markers

Saw Yee

Abstract

Myanmar banana (*Musa spp.*) has many landraces and varieties or cultivars. The present study deals with analyses of genetic diversity and polymorphisms as well as species relationships among the nine banana varieties. A wild banana variety and eight cultivars were based on their DNA finger prints by using PBA (Cytochrome P450 Based Analogues) molecular markers. PBA profiling was conducted in nine banana varieties using eleven primer sets which could be amplified the reproducible bands. The size of amplified products were varied from 350pb to 2700bp. A dendrogram was also constructed based on cluster analysis according to Unweighted Pair Group Mean Average (UPGMA) method.

Key words : Genetic diversity and Polymorphisms, PBA molecular markers, Banana crop improvement, DNA finger prints, Dendrogram , Cluster analysis, UPGMA.

Introduction

Bananas (*Musa* spp.) are originated from South East Asia and Western Pacific area and then introduced into other continents. The banana is ranked as the fourth most important staple food crop in the world.

In higher plants, cytochrome (P450 or CYP) mono-oxygenase enzymes play important roles in oxidative detoxification and biosynthesis of secondary metabolites (Teutsh *et al.* 1993; Ohkawa *et al.* 1998).

Yamanaka *et al.* (2003) used the functional region of mammalian cytochrome P450 gene family to assess genome wide diversity in a range of plant species.

In the present study, PBA (Cytochrome P450 based analogues) molecular markers are used for determining genetic diversity and relation ship in higher plant species determining genetic diversity and relationship in higher plant species including banana plants (*Musa* spp.).

Demonstrator, Dr., Department of Botany, West Yangon University

Materials and Methods

Materials

Nine Myanmar banana varieties were randomly collected from the various locations of Yangon Division and list of these varieties were described on the following Table- 1.

Methods

1. Isolation of Genomic DNA

Genomic DNA from banana sample was extracted according to Fulton *et al.* (1995). Young banana leaves were harvested from each banana variety. About 0.2g of each sample was ground to become very fine powder and was suspended in 750µl of microprep buffer.

Table 1. List of Nine Myanmar banana varieties collected from Yangon division

No.	Scientific name	Vernacular name	Location/ collected Area
1.	*Musa sapientum* L. var. arakenensis	Ya-Khine	Yangon University Campus, VFRDC
2.	*Musa* spp.	Wild banana (Nga-Pyaw-Yine)	YUFL Campus, Mingaladon, VFRDC
3.	*Musa sapientum* L. var. rubra Firm	Shwe-Ni	Yangon University Campus, Hle-Gu, Hmaw-Bi, VFRDC
4.	*Musa chinensis* Sweet	Wet-Ma-Lut	YUFL, Mingaladon,VFRDC
5.	*Musa chiliocarpa* Back	Phee-Gyan	Hle-Gu, Hmaw-Bi,
6.	*Musa doca* Horan.	Pyar-Ye-San	VFRDC
7.	*Musa* spp.	Thu-Nga-Zar	VFRDC
8.	*Musa* spp.	Sait-Noe	VFRDC
9.	*Musa* spp.	Sin-An	Mingaladon, VFRDC

After incubation for one hour at 60^0C the mixture was added and mixed evenly with an equal volume of chloroform : isoamyl alcohol (24:1). The tube was shaken slowly on a rotary shaker. The

mixture was then transferred into an eppendorf tube and was centrifuged about 10,000rpm for 15min.

The aqueous phase was carefully sucked with micropipette and transferred to a new sterile tube. The bottom phase was discarded and the supernatent DNA was mixed with chlorofom : isoamyl alcohol (24:1) solution. This extraction procedure was repeated twice. Following the centrifugation, the aqueous phase was collected and mixed with 300µl of isopropanol by slow inversion. After another round of centrifugation, the precipitated DNA pellet was washed with 70% ethanol and dried over night. After that, the DNA pellet was dissolved in 60µl of double distilled water. Finally, banana DNA samples were loaded on 0.8% mini-agrose gel and were electrophoresed at 100V for 30 minutes. After electrophoresis, the gel was photographed by Polaroid camera with Fuji- 3000 BSS65101 film under the UV transilluminator.

2. DNA Amplification and Gel Electrophoresis

Eleven primer sets out of fifteen PBA primers including three forward primers (CYP1A1F, CYP2B6F, and CYP2C19F) and five reverse primers (CYP1A1R,CYP2B6R,CYP2C19R,HEME2B6, and HEME2C19) were selected for PCR amplification following the protocol by Yamanaka *et al.* (2003) with some minor modifications.

Table 2. Sequence information of PCR primers sets constructed based on known P450 genes

Primer sets		Sequences (5' to 3')						
1	CYP1A1F	GCC	AAG	CTT	TCT	AAC	AAT	GC
2	CYP2B6F	GAC	TCT	TGC	TAC	TCC	TGG	TT
3	CYP2C19F	TCC	TTG	TGC	TCT	GTC	TCT	CA
4	CYP1A1R	AAG	GAC	ATG	CTC	TGA	CCA	TT
5	CYP2B6R	CGA	ATA	CAG	AGC	TGA	TGA	GT
6	CYP2C19R	CCA	TCG	ATT	CTT	GGT	GTT	CT
7	HEME2B6	ACC	AAG	ACA	AAT	CCG	CTT	CCC
8	HEME2C19	TCC	CAC	ACA	AAT	CCG	TTT	TCC

Each experiment was carried out at least twice to determine the extent of reproducibility. The reaction components consisted of 25µM $MgCl_2$, 25mM dNTPs, 25µM primers, 25ng of genomic DNA and 0.1

unit Taq DNA polymerase. The final volume of each amplification reaction was 25µl.

3. DNA Amplification by Polymerase Chain Reaction

A total reaction of 32 cycles was programmed at 94^{0}C for 1 min (Denaturation), 46.5^{0}C, 50^{0}C, 56^{0}C for 2min at each annealing temperature depending on the primer (Annealing) and 72^{0}C for 3min (Extension) in a Thermal Cycler (Applied Biosystem). After completion of 32 cycles, 11µl of amplified samples including loading dye was loaded into 1% min-agarose gel in 0.5 x TBE buffer. Then, the gel was run at 100V for one and half hours. After that, the gel was stained with ethidium bromide for 30min, destained in sterile distilled water for 10 min. Finally, It was photographed under UV light with Polaroid 667 films. The volume of the reaction components were described on the following.

1.10 X PCR Buffer	
(100mM Tris-HCL; pH 8.3; 500mM KCL)	2.5µl
2.Magnisium Chloride ($MgCl_2$) 15mM	2.5µl
3. 2.5mM of dNTPs	2.0µl
4. Primer F (0.2 to 1.0 µM)	1.0µl
5. Primer R (0.2 to 1.0 µM)	1.0µl
6. Taq Polymerase (5 unit)	0.1µl
7. Genomic DNA (25ng)	1:0µl
8. Sterile Distilled Water (up to 25 M)	14.9µl

Total volume of reaction mixture = 25.0µl

The final volume of each amplification reaction was 25µl. The sizes of the amplified fragments were estimated by using size Standard 100bp DNA Ladder Marker.

4. Data Analysis

To estimate the size of PCR products, electrophoregrams were analysed using LabImage 2.7 software programme. Each fragment size was treated as a unit character for analysis and converted to binary

code (1/0). The genetic distance- similarity was estimated based on Pearson Correlation Coefficient (Savekoul *et al.,* 1999)

The amplified DNA markers were scored as 1 (present) and 0 (absent). Most of the strong DNA bands were reproducible and scored for data analysis. Faint bands were not considered for analysis due to their ambiguous nature. A pairwise genetic similarity matrix was prepared based on the genetic similarity in the proportion of DNA fragment shared between the banana samples amplified by the same primer.

Cluster analysis establishing the relationships among the nine banana varieties was performed on the genetic similarity data by using Unweighted Pair Group Mean Average (UPGMA). A dendrogram was constructed from the similarity matrix data of cluster analysis by using Phylip Software Programme.

Results

1. Genetic Polymorphisms Among the Nine Banana Varieties

For the nine Myanmar banana varieties, a total of (174) markers were generated by the eleven selected PBA primer sets. Among them, (142) were polymorphic and the rest (32) markers were monomorphic. The frequency of polymorphisms and monomorphisms were 81.6% and 19.4%. The number of amplified markers for each primer set varied from 2 to 13. The maximum number of polymorphic bands (13) was obtained from the primer set CYP1A1F/CYP2C19R. The average number of polymorphic bands per primer was (9.5) (Figure. 1 to 11).

2. Genetic Similarity, Differences and Relationships Among the Nine Banana Varieties

Similarity indices among the nine banana varieties including eight cultivars and a wild variety were estimated on the basis of all the eleven selected primers which ranged from 0.300 to 0.505. The pairwise similarity matrices computed by cluster analysis programme among nine banana varieties could be used to analyse the genetic similarity and differences among them. Cluster analysis of the genetic

similarity values was conducted to generate the dendrogam indicating genetic relationships among the banana varieties.

The calculations were based on the presence of polymorphic markers among the banana varieties. Based on the dendrogram depicted by the genetic similarities among the varieties, the maximum genetic similarity between Shwe-Ni and Wet-Ma-Lut was (0.505) while the similarity between Pyar-Ye-San and Sait-Noe was (0.498). On the other hand the maximum genetic similarity between Phee-Gyan and Sin-An was (0.479) (Figure. 12 & 13).

According to the dendrogram, Thu-Nga-Zar was clustered with Phee-Gyan and Sin-An despite its genetic similarity between these varieties was (0.430). Likewise, Ya-Khine was more clustered to Pyar-Ye-San and Sait-Noe and the genetic similarity among them was (0.391). Moreover, Ya-Khine, Pyar-Ye-San and Sait-Noe clustering group was also linked with Shwe-Ni and Wet-Ma-Lut cluster group and their similarity index was (0.378). However, the wild banana (Nga-Pyaw-Yine) was very isolated and showed the minimum genetic similarity (0.300) with the other banana varieties.(Figure. 12&13).

Discussion

DNA samples from nine banana varieties were amplified with eleven PBA primer sets and all of them were uniquely distinguishable by at least 1 or 2 polymorphic markers. Among the banana varieties, Thu-Nga-Zar and Nga-Pyaw-Yine showed the maximum amplified bands (95 and 93 out of 174 bands) while Sait-Noe, Ya-Khine and Pyar-Ye-San expressed the minimum amplified (45,49 and 50 out of 174 bands)(Fig.1 to 11).

Based on the presence and absence of polymorphic markers among the banana varieties, a dendrogram was constructed and generated by cluster analysis of genetic similarity showing relationships among these varieties. Cluster analysis based on the genetic similarity values classified the eight banana cultivars into one major group and the wild banana (Nga-Pyaw-Yine) was clearly separated into different group. According to the dendrogram, the major group of cultivated banana varieties were divided into two subgroup, I and II. The subgroup-I consisted five banana cultivars,

namely, Ya-Khine, Pyar-Ye-San, Sait-Noe, Shwe-Ni and Wet-Ma-Lut while subgroup-II comprised three cultivars, namely, Thu-Nga-Zar, Phee-Gyan and Sin-An respectively. The sub-groups were further sub-divided into I-A, I-B, I-A1, I-A2, II-A, II-B, II-A1, II-B1 and so on.

Although the morphotaxonomic traits of Shwe-Ni and Wet-Ma-Lut were not very similar with each other, they were genetically related and clustered in the sub-group I-B. Moreover, the genetic similarity between Shwe-Ni and Wet-Ma-Lut was also congruent with the genome based 15 morphologic diagnostic traits between them. Pyar-Ye-San, Sait-Noe and Ya-Khine were included in the sub-group I-A and they were related with each other. On the other hand, Phee-Gyan, Sin-An and Thu-Nga-Zar were clustered in the subgroup II. Among these varieties, Phee-Gyan and Sin-An were not only similar in their morpho-taxonomic characters but they were high genetic similarity and clustered in the subgroup II-A. Not surprisingly, the wild banana (Nga-Pyaw-Yine) was significantly diversed from eight banana cultivars and clearly observed in the different group (Fig.12 & 13).

It was possible that, the different banana samples of the same variety obtained from different sources might show one or a few differences at DNA level, despite their phenotypic similarity. Hence, the genetic polymorphisms and relationships among the banana varieties were not exactly congruent with their morphotaxonomic characters.

Conclusion

In conclusion, the application of PBA (Cytochrome P450 Based Analogues) molecular markers which informed the genetic polymorphisms and similarity of the banana genotypes in this study based on variations in the functional and genome wide regions could be fulfilled the genetic diversity, relationships and consistent banana classification.

Acknowledgements

My particular thanks and sincene gratitude are to my supervisor Professor Dr. Aung Aung Min, Rector, West Yangon University for suggesting this topic, invaluable advice and overall supervision.

I am also grateful to Dr. Khin Maung Thet, Manager and Dr. Pa Pa Aung, Assistant Manager, Plant Biotechnology Laboratory, Naung Hnapin, Yangon for their guidance and kind help throughout this research.

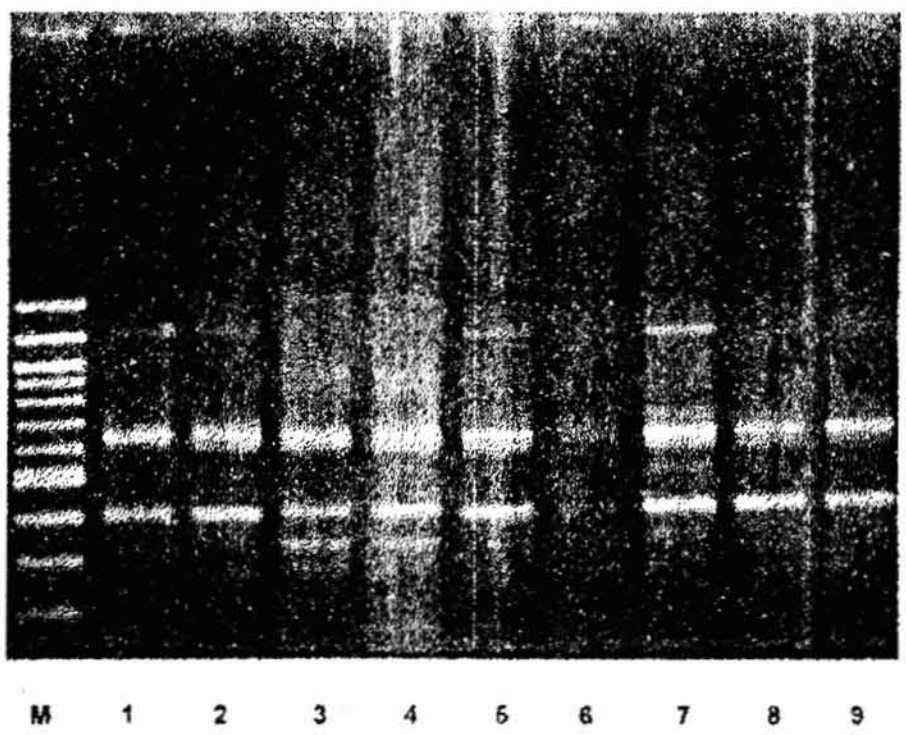


Figure 1. PCR profiles generated by the PBA primer set CYP1A1F/ CYP2C19R

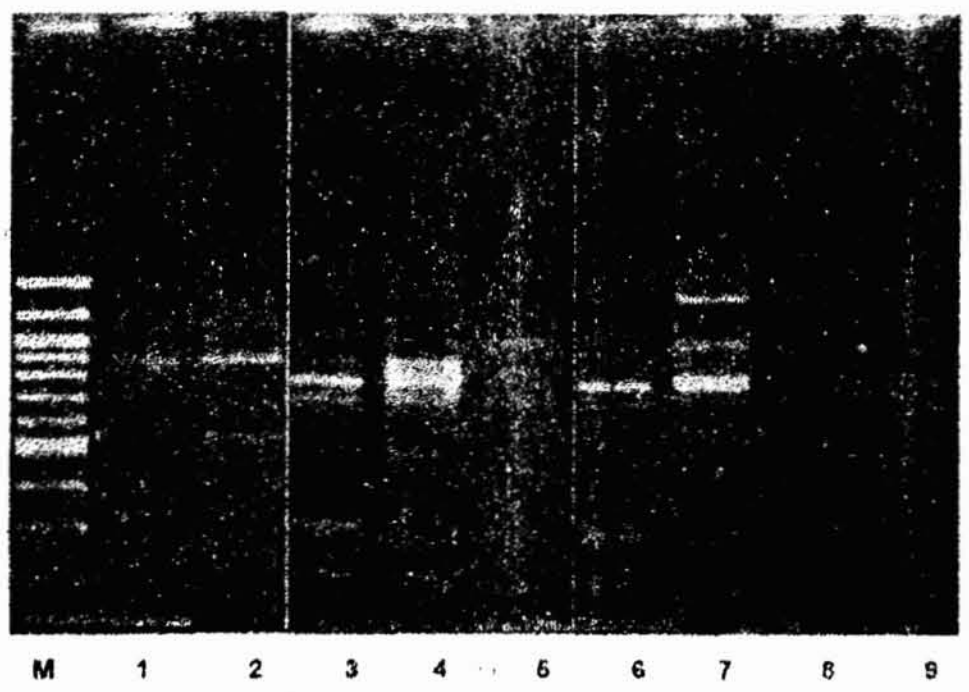


Figure 2. PCR profiles generated by the PBA primer set CYP2C19F/ CYP2C19R

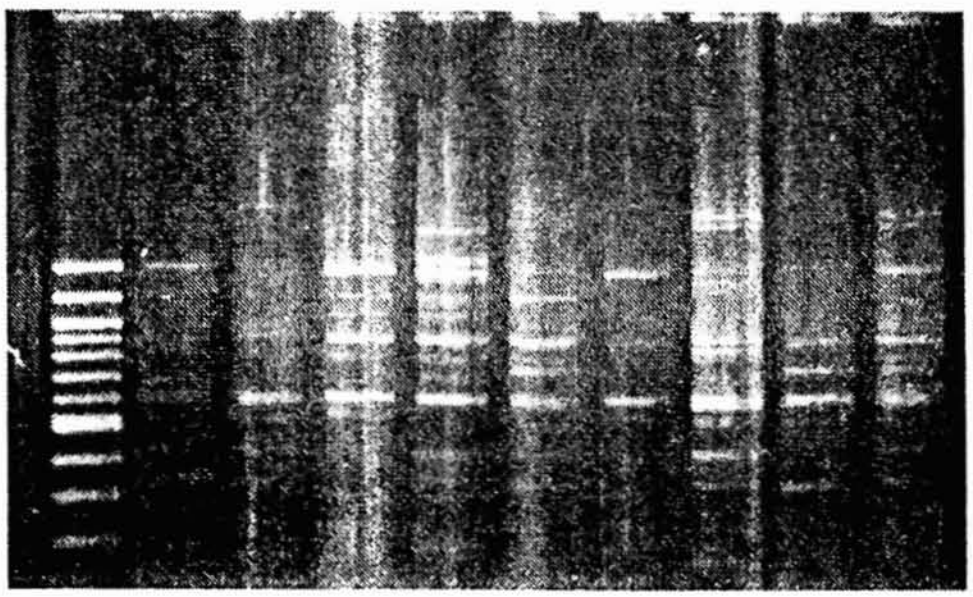


Figure 3. PCR profiles generated by the PBA primer set CYP2B6F/CYP2C19R

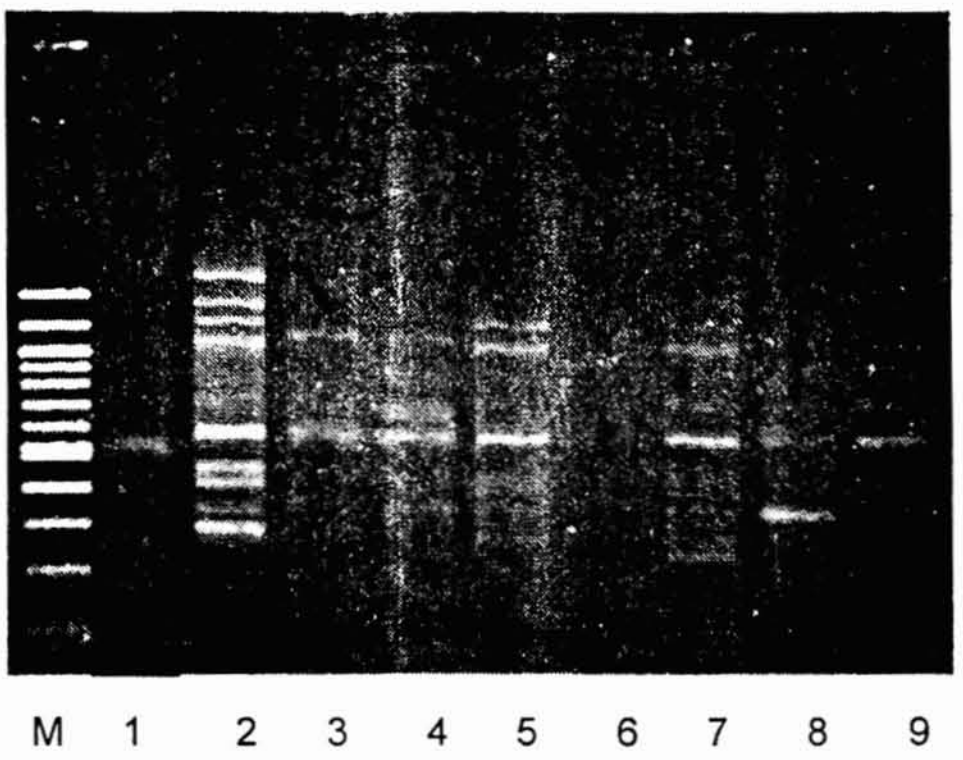
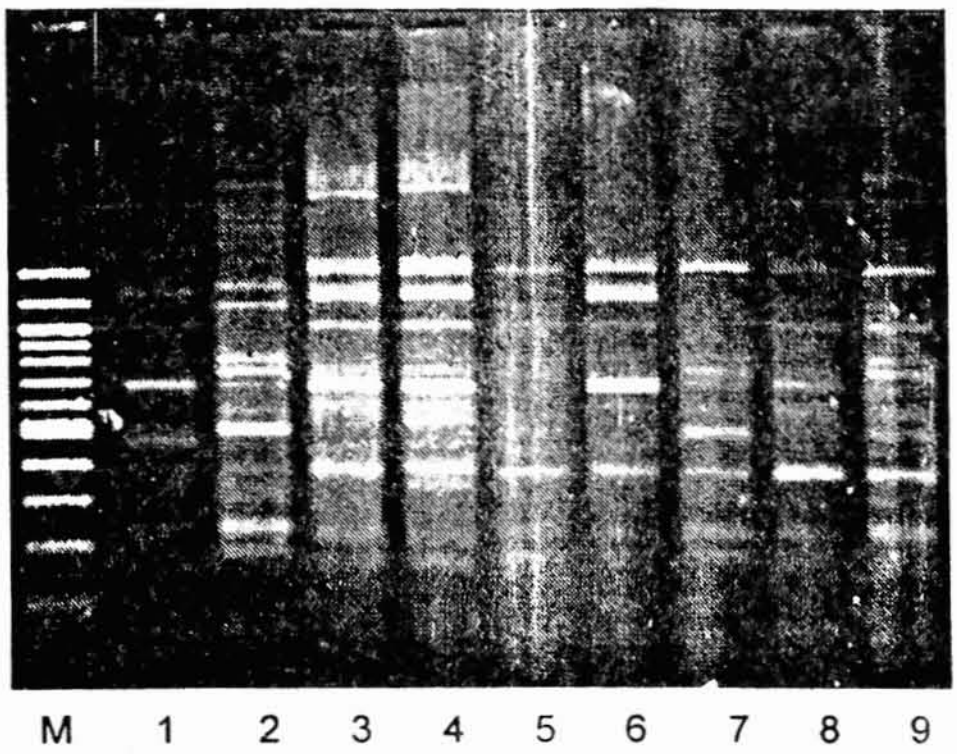
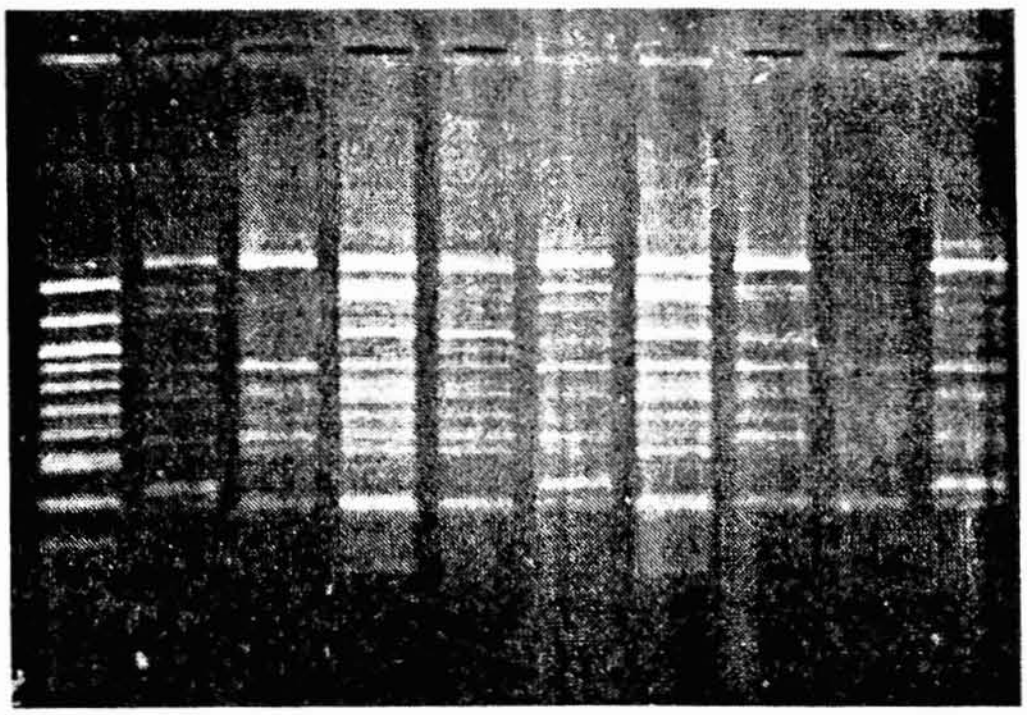


Figure 4. PCR profiles generated by the PBA primer set CYP2C19F/CYP1A1R



M=100bp ladder marker
1= Yakhine
2= Wild Banana
3= Shwe Ni
4= Wet Malut
5= PheeGyan
6= Pyar ye San
7= Thu Nge Zar
8= Sait Noe
9= Sin An

Figure 5. PCR profiles generated by the PBA primer set CYP1A1F/CYP2B6R



M=100bp ladder marker
1= Yakhine
2= Wild Banana
3= Shwe Ni
4= Wet Malut
5= Phe Gyan
6= Pyar ye San
7= Thu Nge Zar
8= Sait Noe
9= Sin An

Figure 6. PCR profiles generated by the PBA primer set CYP2B6F/CYP1A1R

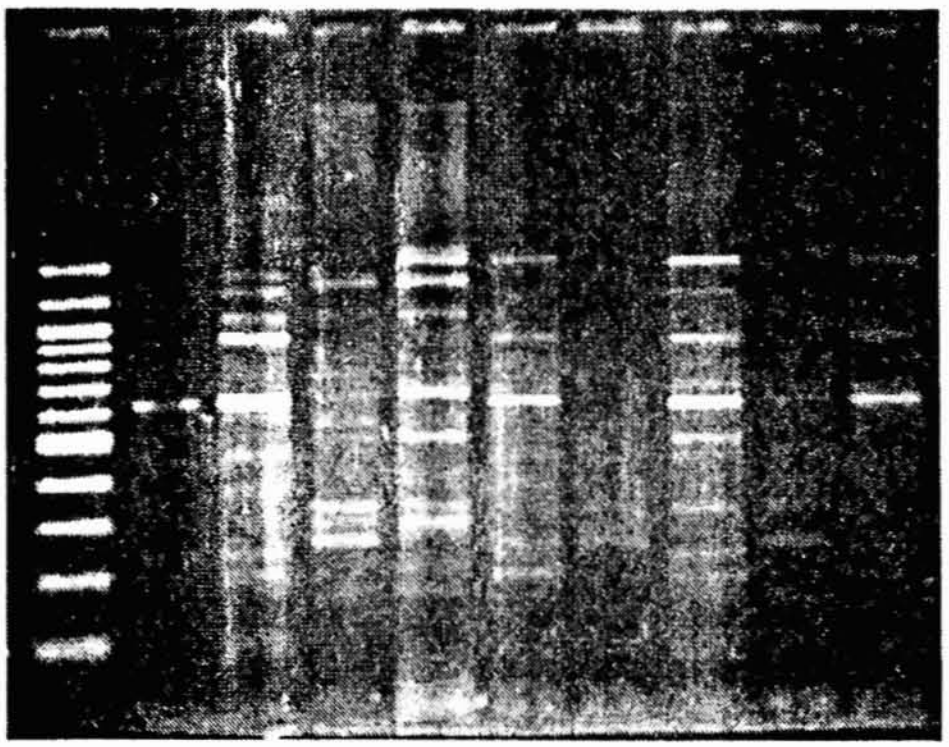


Figure 7. PCR profiles generated by the PBA primer set CYP2B6F/CYP2B6R

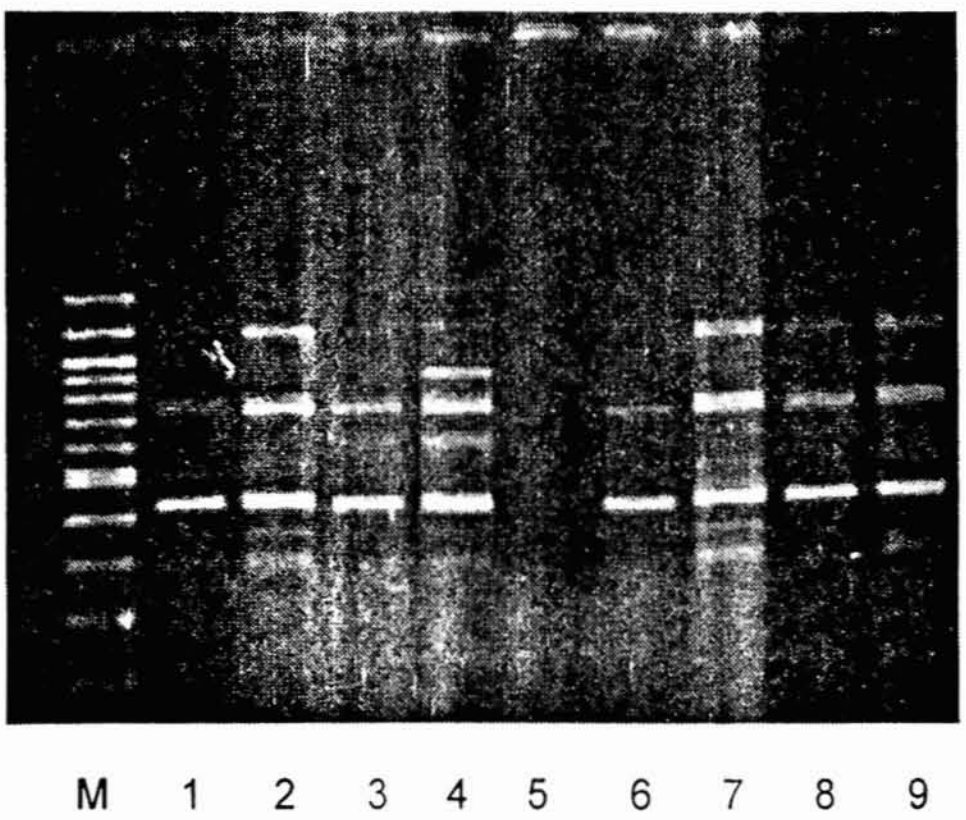


Figure 8. PCR profiles generated by the PBA primer set CYP2B6F/HEME2B6

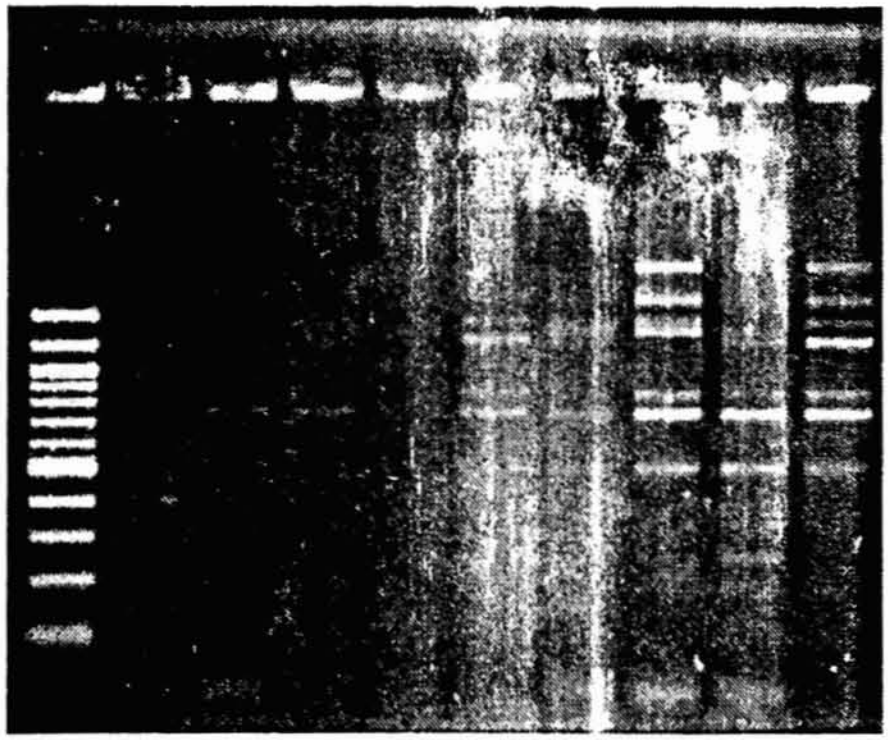


Figure 9. PCR profiles generated by the PBA primer set CYP1A1F/CYP1A1R

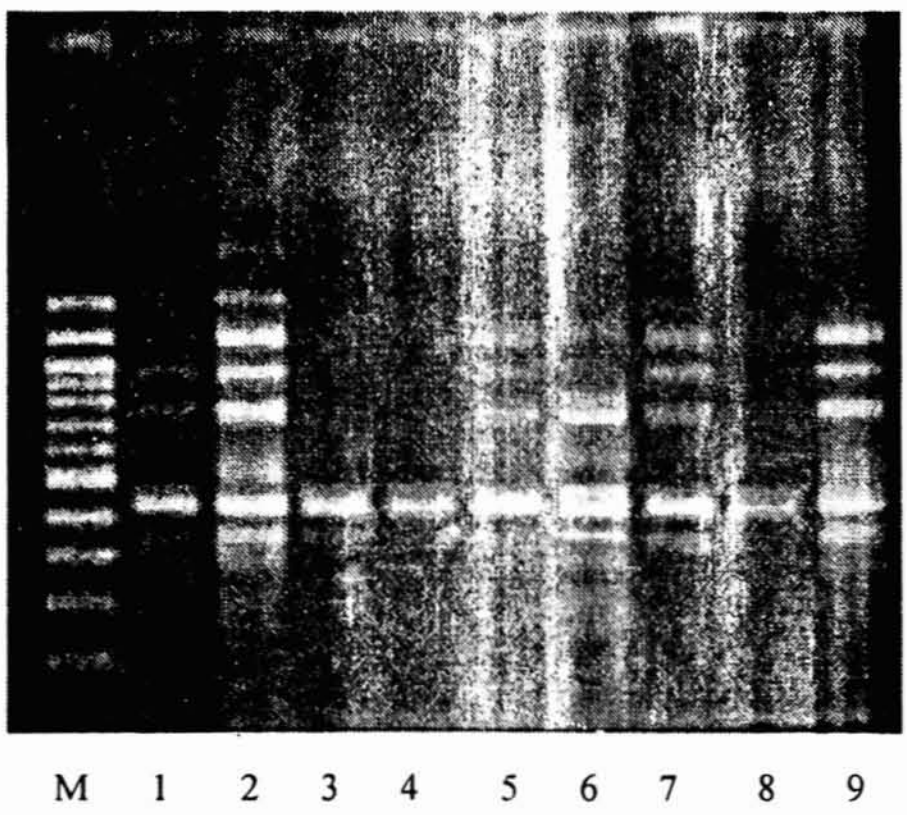


Figure 10. PCR profiles generated by the PBA primer set CYP1A1F/HEME2B6R

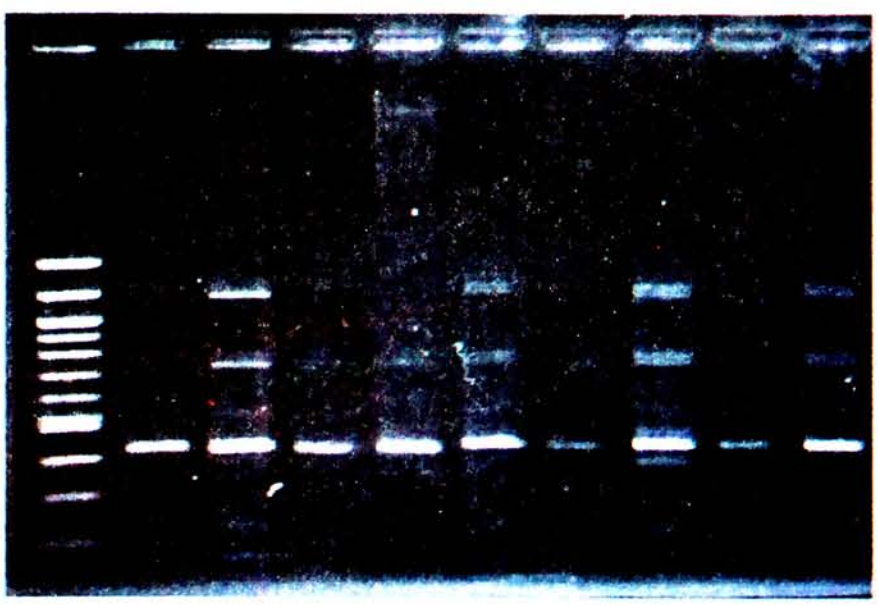


Figure 11. PCR profiles generated by the PBA primer set CYP2C19F HEME2B6R

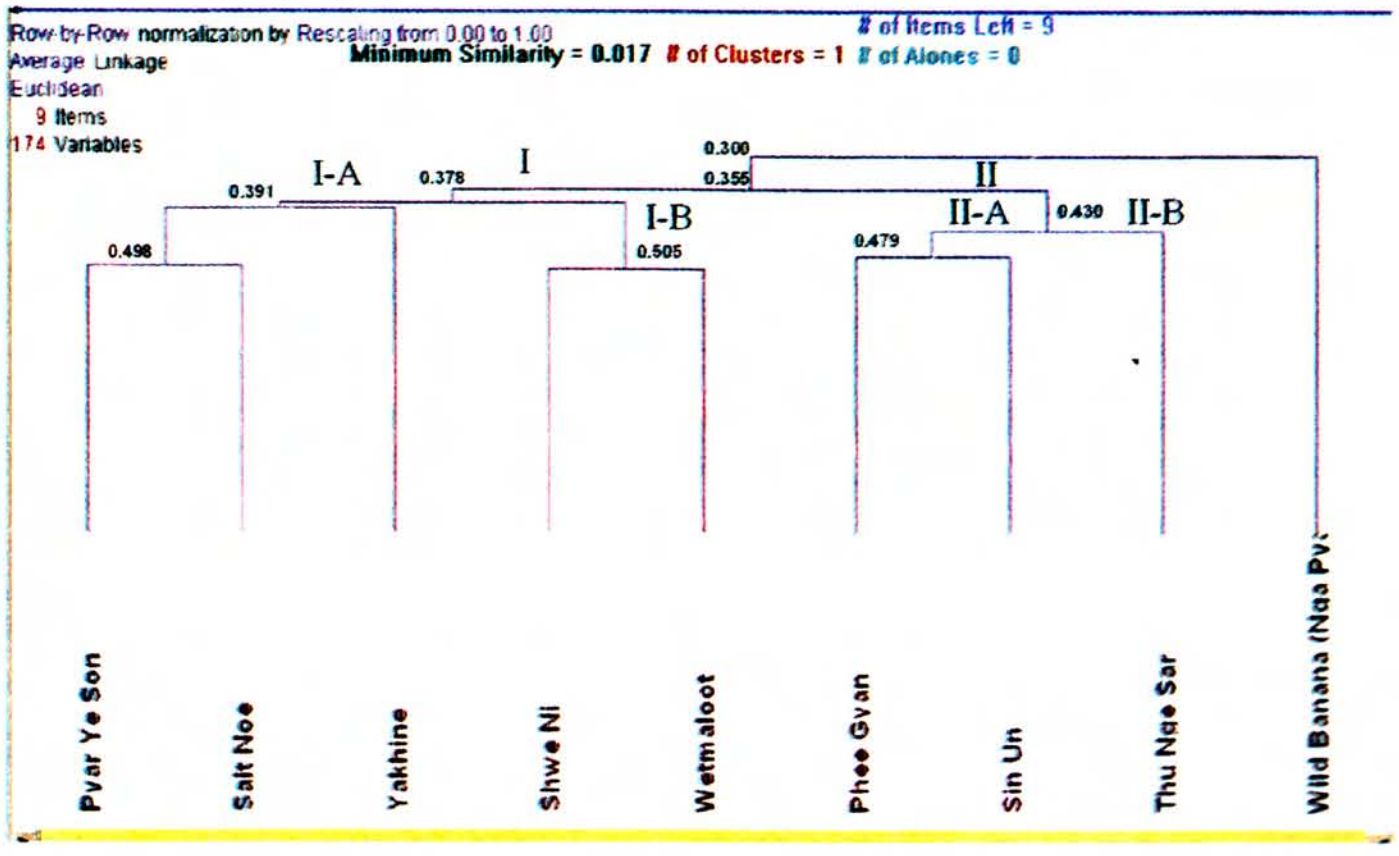


Figure.12. A Dendrogram Showing Relationships Among the Nine Banana Varieties Generated by Cluster Analysis

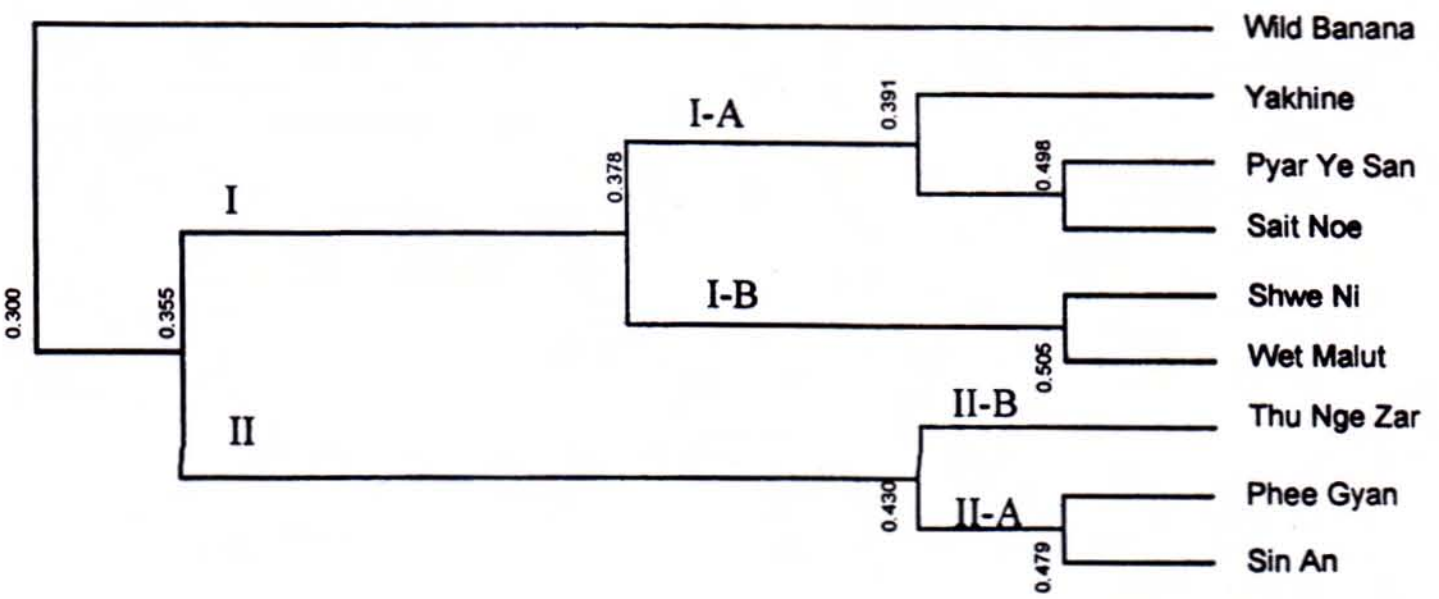


Figure.13. A Dendrogram Showing Relationships Among the Nine Banana Varieties Generated by Cluster Analysis According to Phylip Program

References

Fulton T.M, Chunwongse J, Steven D. T (1995). Micropep protocol for extraction of DNA from tomatoand other herbaceous plants. *Plant Molecular Biology Report* 13 (3): 207-209.

INIBAP Annual Report (2005) *International Network for the Improvement of Banana and Plantain*, Montpellier, France.

Ohkawa H, Imaishi H, Shiota N, Yaamada T, Inui H, Ohkawa Y (1998). Molecular mechanisms of herbicide resistance with sepical emphasis on cytochrome P450 mono-oxigenases. *Plant Biotech* 15:173-176.

Savelkoul P.H.M, Aarts H.J.M, Dehhas J, Dijkshoorn L, Duim B, Osten M, Redemaker J.L.W, Schouls L and Lenstara J.A (1999) Amplified Fragment Length Polymorphism Analysis: The state of an art. *Journal of Clinical Micriobiology*. Vol 37, No.10: 3083-3091.

Teutsch H. G, Hasenfratz M.P, Lesot A, Stoltz C, Garnier J.M, Jeltsch J M, Durst F, Werck-Reichhart D (1993) Isolation and Sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. *Proc. Natl. Acad. Sci.* USA 90: 4102-4106.

Yamanaka S, Susuki E, Tanaka N, Takeda Y, Watanabe J. A and Wanabe K N (2003)Assessment of cytochrome P450 sequences offers a useful tool for determining genetic diversity in higher plant species. *Theot. Appl. Genet.* 108: 1-9.