Cultivos Tropicales, 2004, vol. 25, no. 1, p. 33-38

ISOLATION OF GENOMIC DNAs FROM THE TROPICAL FRUIT TREES AVOCADO, COCONUT, GUAVA AND MANGO FOR PCR-BASED DNA MARKER APPLICATION

Isis M. Ramírez, N. N. Rodríguez✉, Juliette Valdés-Infante, Maricela Capote, D. Becker and W. Rohde

ABSTRACT. With tropical fruit trees, the isolation of genomic DNA with sufficient quality for the application of PCR-based DNA marker technology very often has severe problems due to the presence of inhibitors such as polysaccharides, which inhibit enzymatic DNA processing or polyphenols as inhibitors of PCR reactions. Here, different protocols for DNA extraction and purification were tested with the four tropical fruit trees guava (*Psidium guajava* L.), avocado (*Persea americana* Mill.), mango (*Mangifera indica* L.) and coconut (*Cocos nucifera* L.). The well-established CTAB protocol of Doyle and Doyle yielded excellent DNA templates for PCR amplification with mango and coconut, but not so with guava and avocado. For these latter species, several other extraction protocols also yielded only non-satisfactory results. Modification of the CTAB method with respect to CTAB buffer composition and in combination with reversible adsorption to NucleoSpin columns alleviated the problems encountered with the genomic DNA of both species. The quality of DNA prepared by this procedure allowed AFLP, SSR and ISTR DNA marker analyses in guava and/or avocado.

Key words: DNA, fruit trees, PCR, purification, genetic markers, technology

RESUMEN. En frutales tropicales de porte arbóreo, el aislamiento del ADN genómico con suficiente calidad para ser usado en tecnologías de marcadores moleculares basadas en PCR, posee en muchas ocasiones serios problemas por la presencia de inhibidores, tales como los polisacáridos que inhiben el procesamiento enzimático del ADN o los polifenoles que inhiben las reacciones de PCR. Se probaron diferentes protocolos de extracción y purificación de ADN en cuatro frutales tropicales: guayabo (*Psidium guajava* L.), aguacatero (*Persea americana* Mill.), mango (*Mangifera indica* L.) y cocotero (*Cocos nucifera* L.). El protocolo de Doyle y Doyle logró buenos rendimientos y calidad del ADN para la amplificación (PCR) en mango y cocotero, pero no en guayaba y aguacatero. Para estas dos últimas especies se probaron diferentes técnicas con resultados igualmente insatisfactorios. La modificación del método del CTAB con respecto a la composición del buffer de extracción en combinación con el empleo de columnas de adsorción reversible NucleoSpin, permitió la extracción exitosa del ADN con la calidad suficiente para aplicar las técnicas de AFLP, SSR e ISTR en guayabo y/o aguacatero.

Palabras clave: ADN, árboles frutales, PCR, purificación, marcadores genéticos, tecnología

INTRODUCTION

Guava (*Psidium guajava* L.), avocado (*Persea americana* Mill.), mango (*Mangifera indica* L.) and coconut (*Cocos nucifera* L.) are important fruit trees in tropical and subtropical areas (1). They form an essential part of the human diet and supply the requirements for mineral and vitamins (2, 3). In general, breeding programs with tropical and subtropical fruit trees are inefficient and expensive. Moreover, the genetics for many of these species are poorly understood, and relatively little is known on the size and complexity of their genomes. In order to increase the breeding efficiency with tropical fruits, simple, inexpensive and reliable molecular methods for the characterization of available germplasm such as the application of DNA markers would accelerate such programs (2, 4).

A prerequisite for the application of PCR-based DNA marker technology is the quality of genomic DNA, either for direct PCR amplification (RAPD, SSR, ISTR) or for further manipulation by enzymatic modification such as in AFLP. This problem of isolation of high-quality DNA from tropical woody plants has been recognized as a continuous problem, since contaminants that inhibit the application of molecular methods co-purify with DNA during extractions (5). This report has been focused on four tropical fruit trees of interest and re-examined a variety of protocols for the isolation of DNA that can be subjected to DNA marker techniques. For these four species, efficient, low input protocols are presented for the isolation of high-quality DNA for PCR-based DNA marker application.

Isis M. Ramírez, Especialistas del Centro de Aplicaciones Tecnológicas y Desarrollo Nuclear (CEADEN), Miramar, Playa, Ciudad de La Habana; Dr.C. N. N. Rodríguez, Investigador Titular, Maricela Capote, Investigadora Auxiliar y Juliette Valdés-Infante, Adiestrada del Instituto de Investigaciones en Fruticultura Tropical, Miramar, Playa, Ciudad de La Habana; Profesor W. Rohde y D. Becker, Especialista del Instituto Max-Plack para el mejoramiento genético de las plantas, Colonia, Alemania.

✉ iicif@ceniai.sld.cu

MATERIALS AND METHODS

Plant material. Young leaves of different accessions belonging to the four tropical fruit species: guava, avocado, mango and coconut were used. Samples of the first three were collected from the Tropical Fruticulture Research Institute (IIFT) germplasm collection in Alquízar, Havana and stored at -20°C, before processing. Coconut leaves were harvested in the Baracoa region of eastern Cuba and stored without freezing at room temperature.

Extraction and purification of genomic DNA. Before DNA extraction, all leaf material was cleaned with 70 % ethanol to remove all microorganisms possibly associated with its surface. In general, 5 g of leaf material was cut into pieces, frozen in liquid nitrogen, and ground by mortar and pestle or (preferentially) homogenized to a fine powder using a Waring blender or a standard coffee mill. Unless indicated otherwise (Table I), ground material was incubated in CTAB buffer (2 % cetyltrimethyl ammonium bromide, 0.1 M Tris-HCl pH= 8, 0.02 M ethylendiamine tetra acetic acid, 1.4 M NaCl and 1 % 2-mercaptoethanol) at 60°C for 30 min. before extraction with chloroform:isoamyl alcohol (24:1) and phenol before ethanol precipitation (6). The nucleic acids were then incubated for 30 min. at 37°C in 500 µL of RNAse (50 $\mu g.mL^{-1}$ 1X TE buffer:) + 4.5 mL prewarmed (60°C) 0.2X TE buffer for the digestion of RNA. The composition of 1X TE buffer was: 10 mM Tris-HCl pH = 8, 0.1 mM ethylendiamine tetra acetic acid. Finally, phenol extraction and ethanol precipitation were done (7, 8).

The purified DNA was then carefully redissolved in 500 µL of prewarmed (60°C) 0.2X TE buffer and its purity and integrity analysed using 4 µL of DNA solution on a 0.7% agarose gel after staining with ethidium bromide (0.5 $mg.mL^{-1}$). The approximate quantity was estimated by comparison to DNA markers (Invitrogen Life Technologies).

PCR amplification of genomic DNA. The following DNA marker techniques were used under standard reaction conditions with ^{33}P-labeled PCR primers: Amplified Fragment Length Polymorphism (AFLP) (9), Inverse Sequence-Tagged Repeat (ISTR) (8) and Simple Sequence Repeat (SSR) (10, 11, 12) analyses.

Table I. DNA extraction and purification methods assayed in accessions of four tropical fruit trees

	DNA extraction and purification protocols	Species			
		Guava	Avocado	Mango	Coconut
1	Doyle and Doyle (6) modified by Rohde (7)	Type of plant material: Fresh	Type of plant material: Fresh	Type of plant material: Fresh	Type of plant material: Fresh
2	Doyle and Doyle (6) modified by Rohde (7) + CsCl gradient	Type of plant material: Fresh	Type of plant material: Fresh	__	__
3	QIAGEN DNeasy mini kit for plant DNA extraction (14)	Type of plant material: Frozen	Type of plant material: Lyophilised	__	__
4	QIAGEN Genomic DNA purification from plant leaves. (the two options) (15)	Type of plant material: Frozen	__	__	__
5	Murray and Thompson (17) modified by Lavi (18)	__	Type of plant material: Fresh	__	__
6	Doyle and Doyle (6) modified by Rohde (7). DNA extraction buffer modified by adding PEG_{6000} (1%) and β-mercaptoethanol (50 µL/20 mL). Additionally were included the following enzymes (NOVOZYM) respectively: lyticase (2.5 mg/50 mL), lysis enzymes (2.5 mg/50 mL), cellulase (1 %), esterase (1 %), lipase (1%), gluconase (0.25 %) and a mixture of them	Type of plant material: Frozen	Type of plant material: Frozen	__	__
7	Doyle and Doyle (6) modified by Rohde (7). DNA extraction buffer modified by adding PEG_{6000} (1 %), β-mercaptoethanol (50 µL/20 mL) and cellulase (1 %). There were tested three incubation times (0', 30' and 60')	Type of plant material: Frozen	Type of plant material: Lyophilised	__	__
8	Doyle and Doyle (6) CTAB method modified by K. Aradhya (UC Davis) + NucleoSpin Extract method (19)	Type of plant material: Fresh	Type of plant material: Fresh	__	__

Primers used for PCR amplification were the following:
AFLP:
E32: GAC TGC GTA CCA ATT CAA C
M33: GAT GAG TCC TGA GTA AAA G
M36: GAT GAG TCC TGA GTA AAC C
ISTR:
B3: ATT CCC ATC TGC ACC AAT
F7A: TGC TAG GAC TTT CAC AGA

Aliquots of the reaction mixtures were analysed on 4 % polyacrilamide sequence gels and amplified DNA fragments were made visible by autoradiography (13).

RESULTS AND DISCUSSION

All DNA extraction methods assayed and the type of plant material employed are shown in Table I. The well-established CTAB method (Table I, No. 1) yielded high-quality DNA (Figure 1) for different accessions of mango and coconut. Importantly, the omission of incubating the plant material in CTAB buffer at 60°C for 30 min. greatly improves the quality of DNA: Degradation of DNA does not occur, when the ground plant material is added to CTAB buffer (pre-warmed at 60°C), dispersed by shaking and followed directly by extraction with chloroform and ethanol (or isopropanol) precipitation. In most cases (especially for coconut), the high molecular weight DNA can be fished out of the solution and directly dissolved for RNAse digestion. ISTR and AFLP analyses carried out for both species showed that these DNAs were supporting the modification of genomic DNA (AFLP) and the PCR reactions (AFLP, ISTR) without further purification. In contrast, yield and quality of DNAs in guava and avocado were significantly lower and unreliable using this protocol (data not shown) with only few preparations yielding visible DNA concentrations.

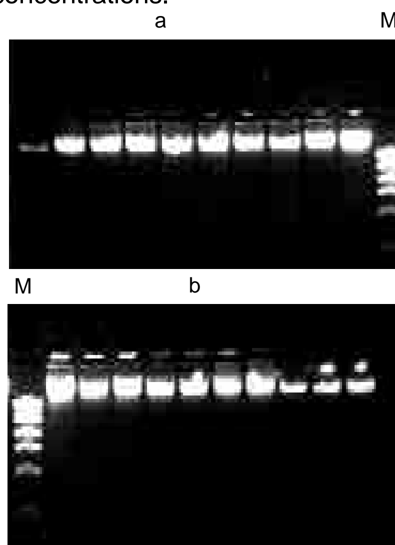

Figure 1. DNAs from mango (a) and coconut (b) accessions as prepared by method no. 1 (Table I). Analysis was performed in a 0.7 % agarose gel. M: 1 kb DNA ladder marker

Despite this fact, ISTR analysis was tested for both species. With avocado, DNA amplification was observed, but results were not reproducible, while the guava DNA preparations did not show any PCR amplification at all (data not shown). Only when these DNAs were further purified by CsCl boyant density gradient centrifugation (Table I, No. 2), the ISTR analysis was successful, although not for every DNA preparation (Figure 2). On the other hand, when avocado DNAs were purified, DNA was visible in the agarose gel (data not shown), but all PCR reactions failed.

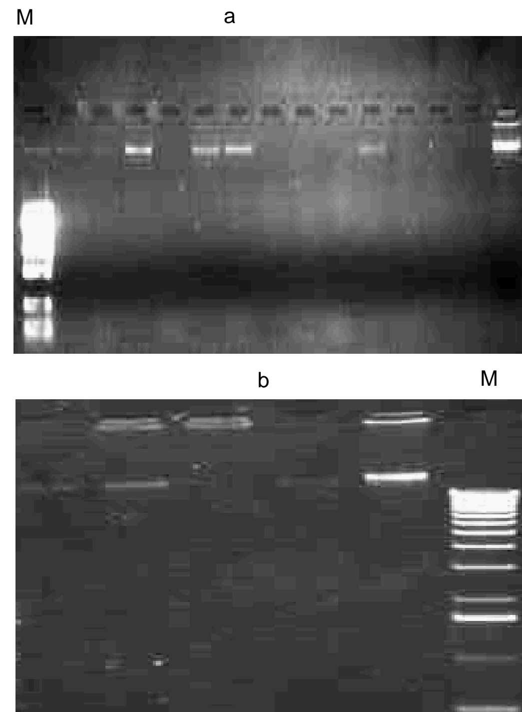

Figure 2. DNAs from guava (a) and avocado (b) accessions as prepared by method no. 1 (Table I). Analysis was performed in a 0.7 % agarose gel. M: 1 kb DNA ladder marker

In view of the unreliable results and the expensive, toxic and time-consuming procedure via gradients, other DNA purification procedures were tested. The QIAGEN DNeasy Mini Kit for DNA isolation from plant tissue (14) (Table I, No. 3) was tested using guava and avocado leaves, but ISTR analyses with the purified DNAs failed due to the absence of PCR amplification. In addition, the two options of CTAB DNA extraction method (Table I, No. 4) (15), followed by anion-exchange column purification (QIAGEN Tip-100) (16), were only partially successful with guava. Similar problems were observed when Murray and Thompson's protocol (17) as modified by Lavi (18) was applied to different avocado accessions (Table I, No. 5). The addition of cell wall-degrading thermo-stable enzymes to CTAB extraction buffer showed the best results with guava, but not with avocado (Table I, No. 6, 7), since ISTR and AFLP techniques were successful when six guava samples were tested (data not shown). Nevertheless, results were not reproducible with other samples extracted.

The best results were obtained for both species using CTAB method with a modification of the extraction buffer composition[1] (Table I, No. 8) in combination with the NucleoSpin Extract method (19) with large amounts of high molecular weight DNA isolated for guava and avocado (Figure 3). This protocol yielded DNA suitable for AFLP as shown in Figure 4. These DNAs also allowed for the application of ISTR and microsatellite (SSR) DNA markers (data not shown).

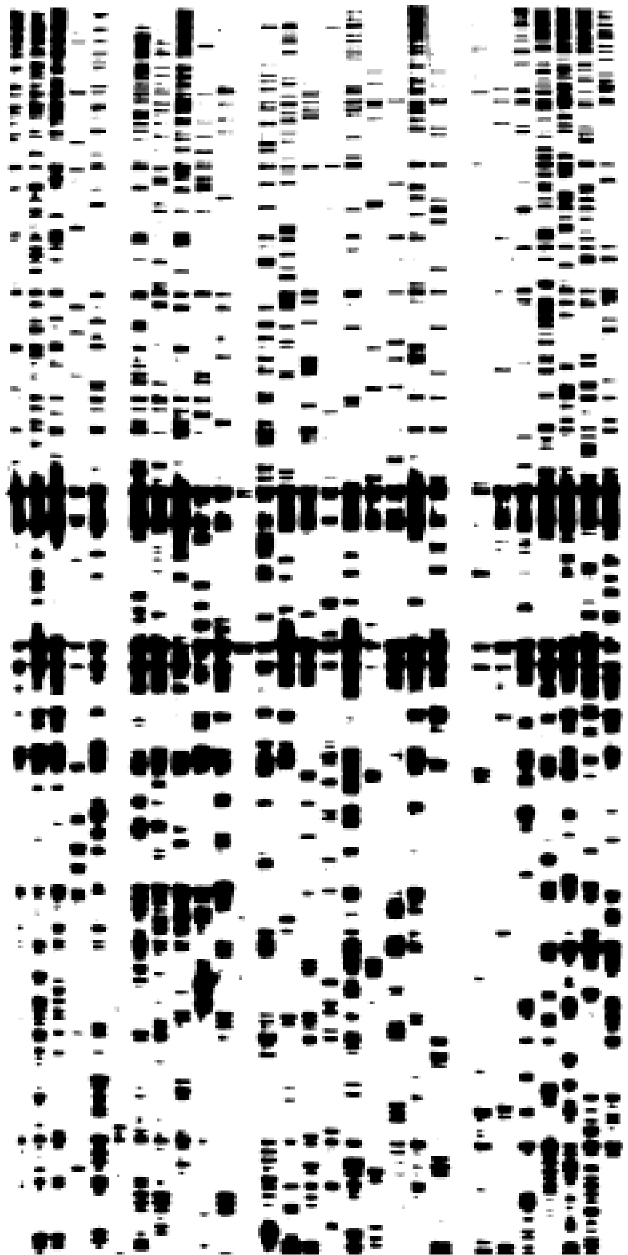

Figure 3. Autoradiogram of an ISTR analysis in guava after DNA purification according to method No. 2 (Table I). For amplification the ISTR primers B3xF7A were used

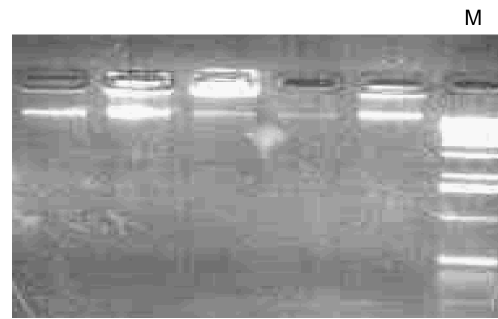

Figure 4. Agarose gel analysis of avocado DNAs as prepared by method No. 2 (Table I). Analysis was performed in a 0.7 % agarose gel. M: 1 kb DNA ladder marker

A standard methodology for DNA isolation from tropical woody plants has been developed (20, 21). Our results, however, demonstrate that a single procedure may not work for different woody species. The fact that CTAB method (6) as modified by Rohde (7) (Table I, No. 1) had been useful for mango and coconut, but not for guava and avocado could be due to the differences existing between plant materials. Guava and avocado leaves are highly sensitive to oxidation resulting in polyphenols (20). A more serious problem is the extremely high content in polysaccharides that co-purify with DNA in standard purification steps. This has already been noted by some authors (5, 22), who described the DNA isolation from tropical trees as notoriously difficult because of high amounts of polysaccharides and many types of secondary metabolites, which form insoluble complexes with nucleic acids when cells are disrupted during extraction. Although De la Cruz reports (10) on an easy and inexpensive general DNA isolation protocol from tropical fruit species including guava and avocado, our work with different avocado accessions could not repeat these results.

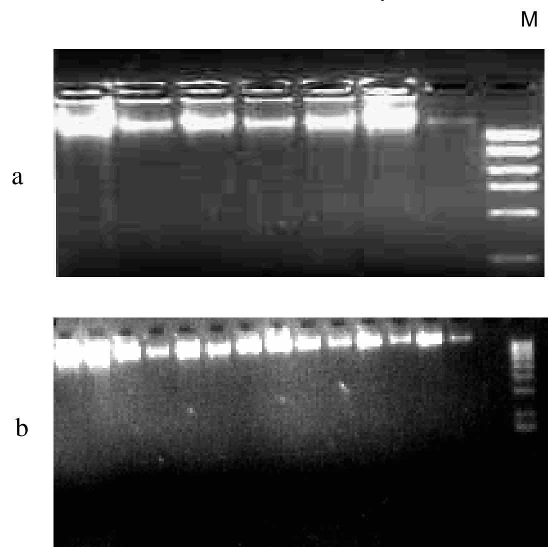

Figure 5. DNAs from guava (a) and avocado (b) accessions as prepared by method No. 8 (Table I). DNAs were subjected to electrophoresis in a 0.7 % agarose gel. M: 1 kb DNA ladder marker

[1] Aradhya, M.K. 2001. UC Davis, personal communication

The success of CTAB method (6) as modified by Aradhya (UC Davis)[1] and followed by the NucleoSpin Extract method (19) over all other methods evaluated in guava and avocado could be explained by the composition of the modified CTAB extraction buffer. This buffer is rich in antioxidant compounds such as: diethyldithiocarbamic acid (DIECA), sodium ascorbate and sodium bisulfite. It is conceivable that these substances play an important role to avoid phenol oxidation and/or the concomitant accumulation of polysaccharide/nucleic acid complexes. It has been reported that a mixture of antioxidant compounds is an effective antioxidant in avocado micropropagation (23, 24).

In conclusion, for mango and coconut the standard Doyle and Doyle CTAB method with slight modifications (7) resulted in high-quality DNA for PCR-based DNA marker technology (Table I, No. 1). In contrast, the modified CTAB buffer in combination with reversible NucleoSpin column adsorption (Table I, No. 8) proved to be the only reliable protocol for the isolation of DNA from polysaccharide-rich fruit trees such as guava and avocado.

ACKNOWLEDGEMENTS

This research was in part funded by the International Bureau, Bonn, Germany (DLR-IB CUB99/002), the DFG/BMZ project Ro 330/10, the PRN/2 – 3/1-2002 project of the Cuban Nuclear Agency of the Ministry of Science, Technology and Environment, and the project 00300213 of the National Program of the Ministry of Science, Technology and Environment (CITMA), Cuba.

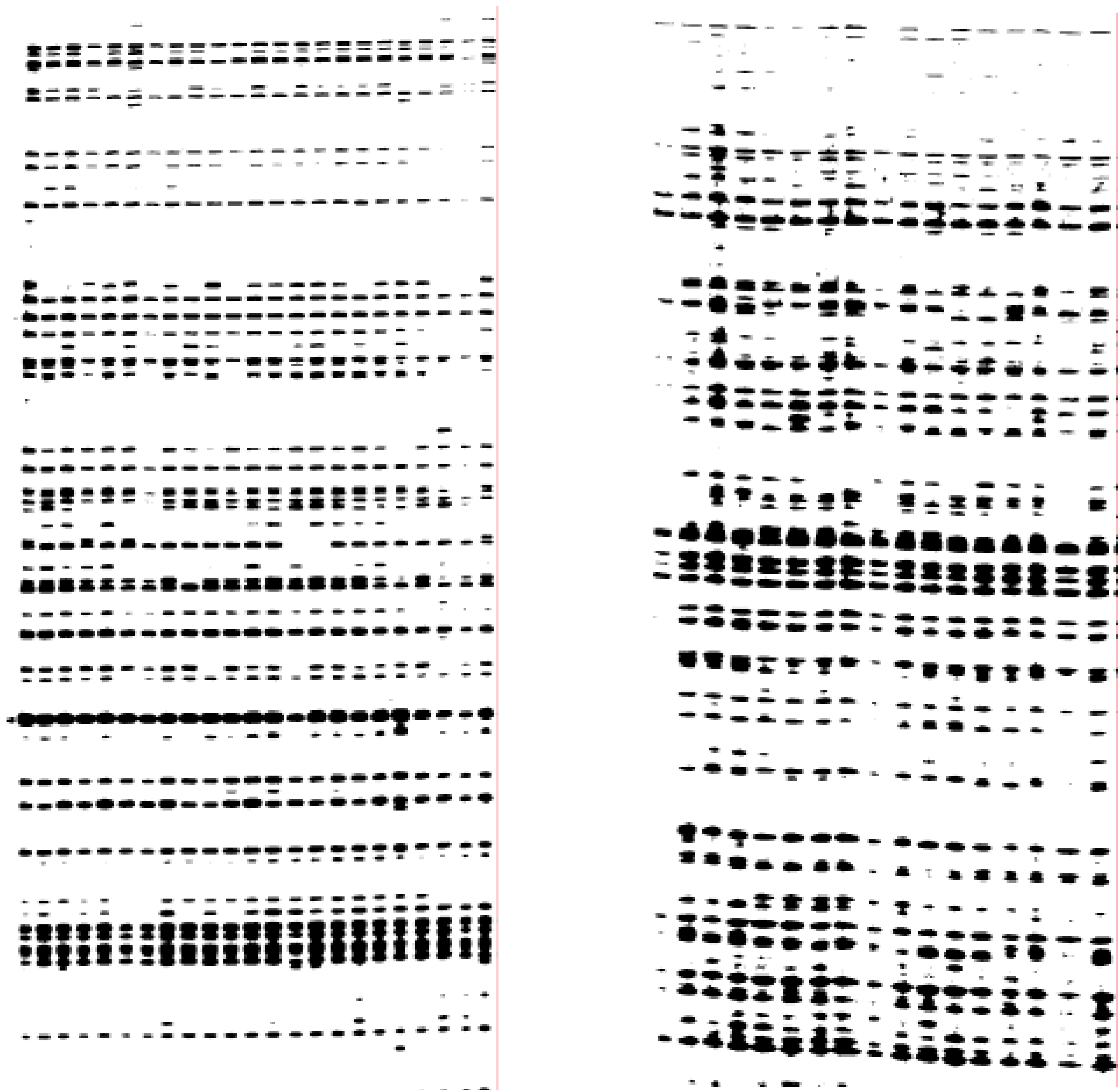

Figure 6. AFLP analysis of DNAs from guava (a) and avocado (b) accessions. DNAs were prepared according to method No. 8 (Table I) and amplified with AFLP primers E32xM33 (a) and E32xM36 (b)

REFERENCES

1. Nakasone, H. Y. and Paull, R. E. Tropical Fruits. Wallingford : CAB International, 1998.
2. Henry, R. J. Practical application of molecular markers to tropical and subtropical species. *Acta Hort.*, 1998, vol. 461, p. 107-111.
3. Péres, R. M. and Pierre, D. Significant applications of biotechnology in tropical and subtropical species. *Acta Hort.*, 1998, vol. 461, p. 31-40.
4. Mhameed, S. /*et al.*/. Genetic relationships within avocado (*Persea americana* Mill.) cultivars and between *Persea* species. *Theor. Appl. Genet.*, 1997, vol. 94, p. 279-286.
5. Guillermaut, P. and Marechal-Drouart, L. Isolation of plant DNA: a fast, inexpensive and reliable method. *Plant Molecular Biology Reporter*, 1992, vol. 10, p. 60-65.
6. Doyle, J. J. and Doyle, J. L. Isolation of plant DNA from fresh tissue. *Focus*, 1990, vol. 12, p. 13-15.
7. Rohde, W. /*et al.*/. Genetic analysis of *Cocos nucifera* L. by PCR amplification of spacer sequences separating a subset of *copia*-like *Eco*RI repetitive elements. J. Genet. & Breed.,1995, vol. 49, p. 179-186.
8. Rohde, W. Inverse sequence-tagged repeat (ISTR) analysis, a novel and universal PCR-based technique for genome analysis in the plant and animal kingdom. *J. Genet & Breed.*, 1996, vol. 50, p. 249-261.
9. Vos, P. /*et al.*/. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.*, 1995, vol. 23, p. 4407-4414.
10. Litt, M. and Luty, J. A. A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.*, 1989, vol. 44, p. 397-401.
11. Tautz, D. Hypervariability of simple sequences as a general source of polymorphic DNA markers. *Nucleic Acids Res.*, 1989, vol. 17, p. 6463-6471.
12. Weber, J. K. and May, P. E. Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.*, 1993, vol. 44, p. 388-396.
13. Ramírez, I. M. /*et al.*/. DNA polimorphic in Cuban varieties of avocado (*Persea Americana* Mill.) as detected by Inverse Sequence Tagged Repeat (ISTR) analysis. *Cultivos Tropicales*, 2002, vol. 23, no. 3, p. 85-88.
14. QIAGEN. Dneasy ™ Plant Mini Handbook for DNA isolation from plant tissue. Germany, 1999, p. 4-22.
15. QIAGEN. Genomic DNA Purification from Plant Leaves. Genomic PB. Germany, 1995.
16. QIAGEN. QIAGEN® Plasmid Purification Handbook. Germany. 1999, p. 3-78.
17. Murray, M. G. and Thompson, W. F. Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Res.*, 1980, vol. 8, p. 4321-4325.
18. Lavi, U. /*et al.*/. Application of DNA fingerprints for identification and genetic analysis of avocado. *J. Amer. Soc. Hort. Sci.*, 1991, vol. 116, p. 1078-1081.
19. Macherey & Nagel. NucleoSpin® Extract 2 in 1 Protocol. *Germany*, 2002, p. 3-13.
20. De la Cruz, M.; Whitkus, R. and Mota-Bravo, L. Tropical fruit tree DNA isolation and amplification. *Molecular Ecology*, 1995, vol. 4, p. 787-789.
21. Csaikl, U. /*et al.*/ Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. *Plant Molecular Biology Reporter*, 1998, vol. 16, p. 69-86.
22. Pandey, R. N.; Adams, R. P. and Flournoy, E. Inhibition of random amplified polymorphic DNAs (RAPDs) by plant polisaccharides. *Plant Molecular Biology Reporter*, 1996, vol. 14, No.1, p. 17-22.
23. Rodríguez, N. N; Capote, M. and Zamora, V. Cultivo *in vitro* del aguacatero (*Persea americana* Mill.). *Revista Chapingo. Serie Horticultura*, 1999 (Número especial), p. 233-238.
24. Capote, M.; Rodríguez N. N. and Blanco, M. Cultivo *in vitro* del aguacatero (*Persea americana* Mill.). *CITRIFRUT*, 2001, vol. 19, no. 1, p. 2-8.

Recibido: 13 de febrero de 2003
Aceptado: 4 de julio de 2003

DIPLOMADOS

Precio: 2000 USD

Incremento en la producción de las áreas afectadas por la sequía

Coordinador: Dra.C. María C. González Cepero
Duración: 1 año

SOLICITAR INFORMACIÓN

Dr.C. Walfredo Torres de la Noval
Dirección de Educación, Servicios Informativos y Relaciones Públicas
Instituto Nacional de Ciencias Agrícolas (INCA)
Gaveta Postal 1, San José de las Lajas,
La Habana, Cuba. CP 32700
Telef: (53) (64) 6-3773
Fax: (53) (64) 6-3867
E.mail: posgrado@inca.edu.cu