



# OPTIMIZATION OF THE PROTOCOLS OF EXTRACTION OF ADN AND OF THE MOLECULAR MARKER TYPE RAPD IN ANONÁCEAS

## Optimización de los protocolos de extracción de ADN y del marcador molecular tipo RAPD en Anonáceas

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**ABSTRACT.** The molecular techniques need of protocols that allow determine levels of genetic change inside the populations in different environmental conditions. So much the optimization of the isolation of the DNA, as that of the working conditions of the amplifications, they are fundamental to reach the success of the molecular analyses, therefore the present research has like objective: optimize protocols of extraction of DNA and of the molecular marker type RAPD (Random Amplified Polymorphic DNA) in Anonáceas. For the DNA extraction were used the Kit Nucleon PHYTOpure and DNeasy® of QIAGEN. The working conditions of the protocol of amplification were fitted and changed the concentrations of DNA and of the used chokers. Followed by this, a test was realized with 10 chokers of the series OPH and five of the series OPA, to select more polymorphisms. The first results obtained with the Kit Nucleon PHYTOpure showed a DNA of low quality, due to the high phenolization of the vegetable material, not like that with the Kit DNeasy® of QIAGEN, who allowed obtain a DNA of quality, purity and homogeneity to an approximate concentration of 30 ng  $\mu\text{L}^{-1}$ . The biggest amplification products were obtained with 3 ng  $\mu\text{L}^{-1}$  of choker and 2 ng  $\mu\text{L}^{-1}$  of DNA. Four chokers that presented major polymorphism were OPA-16, OPH-03, OPH-13 and OPH-18. The results of this research allowed optimize the working conditions of the technical RAPD for the characterization of the collection *ex-situ* of Anonáceas under our environmental conditions.

**RESUMEN.** Las técnicas moleculares requieren de protocolos que permitan determinar los niveles de variación genética, dentro de las poblaciones en diferentes condiciones ambientales. Tanto la optimización del aislamiento del ADN, como el de las condiciones de trabajo de las amplificaciones, son fundamentales para alcanzar el éxito de los análisis moleculares, por lo que la presente investigación tiene como objetivo: optimizar los protocolos de extracción de ADN y del marcador molecular tipo RAPD (Random Amplified Polymorphic DNA) en Anonáceas. Para la extracción de ADN se utilizaron los Kit Nucleon PHYTOpure y DNeasy® de QIAGEN. Se ajustaron las condiciones de trabajo del protocolo de amplificación, en el que se variaron las concentraciones de ADN y las de los cebadores utilizados. Seguido de esto, se realizó un testaje con diez cebadores de la serie OPH y cinco de la serie OPA, para seleccionar los más polimórficos. Los primeros resultados con el Kit Nucleon PHYTO pure mostraron un ADN de baja calidad, debido a la alta fenolización del material vegetal, no así con el Kit DNeasy® de QIAGEN, que permitió obtener un ADN de calidad, pureza y homogeneidad a una concentración aproximada de 30 ng  $\mu\text{L}^{-1}$ . Los mayores productos de amplificación se obtuvieron al usar 3 ng  $\mu\text{L}^{-1}$  de cebador y 2 ng  $\mu\text{L}^{-1}$  de ADN. Los cuatro cebadores que presentaron mayor polimorfismo fueron OPA-16, OPH-03, OPH-13 y OPH-18. Los resultados de esta investigación permitieron optimizar las condiciones de trabajo de la técnica RAPD para la caracterización de la colección *ex situ* de Anonáceas bajo nuestras condiciones ambientales.

**Key words:** DNA isolation, *Annonaceae*, RAPD

**Palabras clave:** aislamiento de ADN, *Annonaceae*, RAPD

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## INTRODUCTION

The exploitation of plants has encouraged farmers to select and improve lines, crops or certain species with desired characteristics (1). Nowadays, molecular biology techniques have been used to obtain specific genetic markers for each species, to find polymorphic differences and to provide the necessary information for the identification of plant materials under study<sup>A</sup> (2, 3, 4).

Molecular markers allow estimation of genetic distance, identification and discrimination of populations, varieties, pure lines and hybrids, they also establish kinship relationships and locate and identify regions of DNA that affect quantitative traits (5, 6). The analysis of genetic markers is very useful because of the polymorphism they detect, Mendelian inheritance without epistasis; that is to say, without interaction between the genes, the insensitivity to the environmental factors or the development of the plant and its easy identification and co-dominance (7, 8).

In the identification of a species, the first step to develop is the isolation of DNA or genetic material, which must be sufficiently pure for its manipulation and amplification. Currently there are different commercial systems that allow the extraction of DNA from plant material. However, we must not rule out the use of other techniques that have been proposed in order to reduce the interference caused by the high content of polyphenols or polysaccharides in certain species such as Agaves and Anonaceae. This interference in the sample brings with it the decrease in the purity and performance of the DNA<sup>A,B</sup>.

Molecular techniques require protocols to determine the levels of genetic variation within populations, under different environmental conditions, and the characterization of plant genetic resources. It is essential to have adequate DNA isolation and purification methods that allow the application of various molecular biology techniques as well as efficient protocols for the amplification of DNA by markers (9).

There are two important alternatives for the conservation of plant genetic resources (RFG) in situ and ex situ, currently the latter is highlighted (10).

The success of this type of conservation depends on the accessibility of the materials conserved in them and the correct characterization of their germplasm (11). This facilitates the rational management of collections and breeding programs, the proper selection of parents for cross-breeding strategies and the construction of genetic maps. In addition, it eliminates duplications and ensures the correct identification of its accessions (12, 13).

In Cuba, the only *ex situ* collection of *Anonáceas* that exists is located in the Experimental Station of Fruits from Alquizar, belonging to the Tropical Fruit Research Institute (IIFT). This family includes an important group of tropical fruits very desired by the population. However, established commercial plantations have been significantly reduced, their cultivation has been limited for years, so they are only found almost exclusively in the yards and gardens of rural villages and in some cities (14, 15).

In the country there are no reports on the use of RAPD molecular markers for the characterization of the *Annonaceae* family. The success of both molecular and other techniques lies in the adjustment of working conditions, from DNA isolation to PCR conditions, for this reason, the present research aims to optimize the extraction protocols of DNA and RAPD molecular marker in *Anonaceae*.

## MATERIALS AND METHODS

The present work was made from samples of plants from the *ex situ* collection of the *Annonaceae* family of Cuba. The collection is preserved in the Experimental Station of Fruits from Alquizar, belonging to the Tropical Fruit Research Institute (IIFT), located at latitude 22° 47' N and longitude 82° 31' West at 110 m a. s. l. It is planted on a typical Red Ferralitic soil with pH around 5,5-6,5 and flat topography, at a planting distance of 7 x 7 m. It does not present irrigation, so it is under dry conditions, without fertilization and the trees have different ages (plants of replanting and until more than 15 years).

### ISOLATION OF DNA

For the optimization of the DNA amplification protocol with the type molecular marker, the quality and homogeneity of the DNA of the materials were guaranteed. In the DNA isolation, two protocols were used: the NucleonPHYTOpure Extraction Kit (without columns) from the Amersham firm and the DNeasy<sup>®</sup> extraction kit from the commercial firm QIAGEN (with columns, Figure 1). As a plant material, young leaves of visibly healthy plants (Table I).

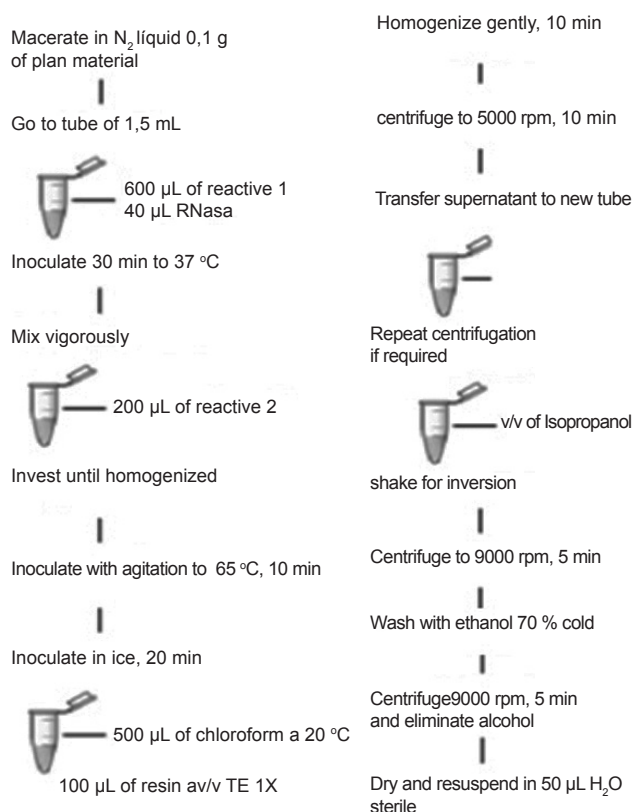
<sup>A</sup>Feria, R.I.A. *Clonación y caracterización de fragmentos RAPD y loci microsatélite, asociados a Psidium guajava L. cultivada en 4 estados de la República Mexicana*. [en línea] [Tesis de Doctorado], Universidad Autónoma Metropolitana, México, 2008, [Consultado: 28 marzo 2015], Disponible en: <http://148.206.53.84/tesiuami/UAMI14289.pdf>.

<sup>B</sup>Herrera, R.J.L. *Optimización de protocolos para la extracción de ADN y uso del marcador SCAR ISPJ1 en Piñón (Jatropha curcas L.)* [en línea] [Tesis presentada en opción al título de Ingeniero Agropecuario], Escuela Agrícola Panamericana El Zamorano, Honduras, 2010, 66 p., [Consultado: 28 marzo 2015], Disponible en: <http://bdigital.zamorano.edu/handle/11036/601>.

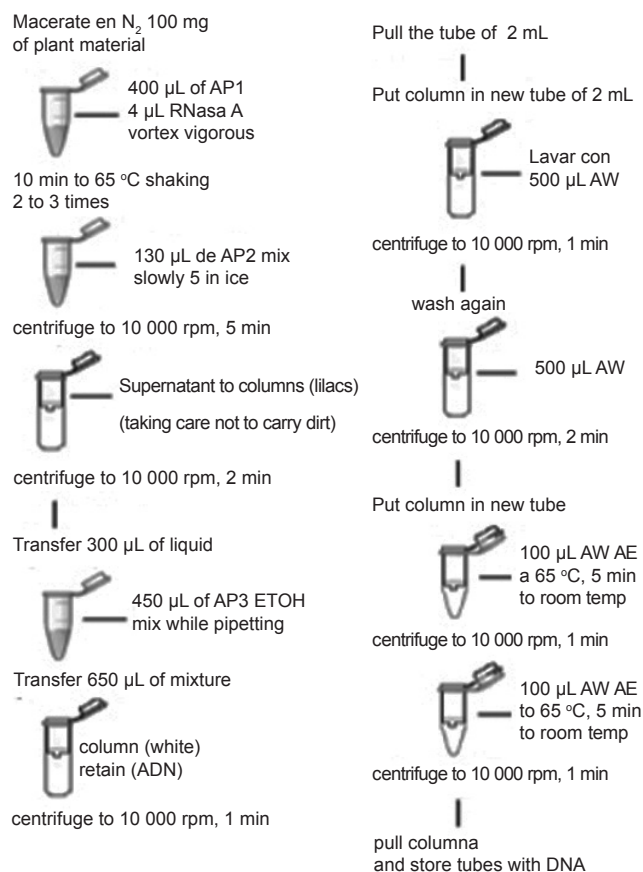
**Table I. Accessions of the *Annonaceae* family from the national *ex situ* collection**

Number of identification	Vegetal material	Genre	Specie
1A	Rollinia	<i>Rollinia</i>	<i>deliciosa</i>
2A	Mamón	<i>Annona</i>	<i>reticulata</i>
3A	Bagá	<i>Annona</i>	<i>glabra</i>
4A	Bagá	<i>Annona</i>	<i>glabra</i>
5A	Bagá	<i>Annona</i>	<i>glabra</i>
6A	Guanábana	<i>Annona</i>	<i>muricata</i>
7A	Híbrido	?	?
8A	cv.?	?	?
9A	Mamón	<i>Annona</i>	<i>reticulata</i>
10A	Mamón	<i>Annona</i>	<i>reticulata</i>
11A	Mamón	<i>Annona</i>	<i>reticulata</i>
12A	Anon	<i>Annona</i>	<i>squamosa</i>
13A	Anon	<i>Annona</i>	<i>squamosa</i>
14A	Anon	<i>Annona</i>	<i>squamosa</i>
15A	Anon	<i>Annona</i>	<i>squamosa</i>

**A) Kit of extraction Nucleon PHYTO pure**



**B) Kit of extraction DNeasy® QIAGEN**



**Figure 1. Scheme of the genomic DNA isolation protocol using the Nucleon PHYTOpure and DNeasy® QIAGEN Kits**

The quality and homogeneity of genomic DNA was verified on 0,8 % agarose gel in 1X TBE buffer, which was run at 100 V for 30 min. To visually estimate the DNA concentration the gel was observed in an ultraviolet light transilluminator.

## DNA AMPLIFICATION WITH TYPE RAPD

### MOLECULAR MARKER

To establish the working conditions in the optimization of the protocol to be used in DN amplification using the RAPD molecular marker, some of the components of the master mix were modified for a total volume of 30  $\mu\text{L}$ . Initially, different concentrations of genomic DNA (30 ng  $\mu\text{L}^{-1}$ ) (1, 2 and 4 ng  $\mu\text{L}^{-1}$  reaction) were tested, once the most appropriate concentration was adjusted, that of the primer (100  $\mu\text{M}$ ) 1, 2 and 3 ng  $\mu\text{L}^{-1}$ ) (Table II).

**Table II. Components of the Master Mix in a final volume of 30  $\mu\text{L}$**

Components of the mixture	Concentrations ( $\mu\text{L}$ )
Master Mix	15
Taq	0,2
Primer	1, 2 o 3
ADN	2, 4 y 8
H <sub>2</sub> O	Adjusts to volume

In PCR amplification, a thermocycler of the TECHNE brand model TC-3000 was used in which a program of approximately three hours duration was followed where the initial denaturation was 5 min at 94 °C; followed by 35 cycles each denaturation cycle of 30 s at 94 °C, hybridization at 36 °C for 30 s and extension at 72 °C for 1 min, the program ends with a final extension cycle at 72 °C for 5 min.

The generated RAPD amplification products were separated by horizontal electrophoresis in 1,5 % agarose gels (1X TBE buffer). Electrophoretic chamber with electric conduction buffer was run at the same concentration at 80 V and molecular weight marker (1Kb DNA Ladder INVITROGEN®)

These gels were stained with 0.75  $\mu\text{g mL}^{-1}$  ethidium bromide and observed in an ultraviolet light transilluminator (312 nm) for visualization of the amplified fragments.

Once the DNA amplification protocol was standardized using the molecular marker RAPD, we proceeded to the selection of the most polymorphic primers for the subsequent characterization of the ex situ collection of *Anonaceae*. For this, a total of 15 decameric primers (Table III) of Operon Technologies (Alameda CA, USA) were tested with the DNA of three different genotypes of the accessions under study.

The master mix was the one that allowed better amplification of the DNA, where in a total volume of 30  $\mu\text{L}$  there were 15  $\mu\text{L}$  of Master mix (2X), 3  $\mu\text{L}$  of primer (10 pmoles  $\mu\text{L}^{-1}$ ); 0,2  $\mu\text{L}$  Taq polymerase (5 U  $\mu\text{L}^{-1}$ ) (Ferments), 30 ng genomic DNA and to be completed with water. In the amplification of the PCR for the separation of the RAPD products and their visualization, the methodology previously described was followed. From the amplified products, 300 pixel/ inch photographs were taken and manually analyzed to select primers with the highest polymorphism detected (PCR reactions were repeated twice).

## RESULTS AND DISCUSSION

### ISOLATION OF DNA

In selecting the appropriate DNA isolation protocol, the desired results were not obtained with the Nucleon PHYTOpure Extraction Kit (Figure 2). This negative result could be due to the high levels of phenolization that were reached in the majority of the accessions, mainly in the samples of the species *A. reticulata*. Similar phenolization results have been obtained by extracting the DNA of *Anonaceae* plants preserved in the Ecuador<sup>c</sup> germplasm bank and in *Annona senegalensis* Pers collected in a forest of Kachia Kaduna, Nigeria (15).

<sup>c</sup> Escribano, M. *Caracterización de marcadores moleculares para la identificación de genotipos, estudio de diversidad genética y mejora del chirimoyo (A. cherimola Mill.)*. [Tesis de Doctorado], Facultad de Ciencias, departamento de microbiología. Universidad de Málaga, España, 2006, 139 p.

**Table III. Primers RAPDs used for the amplification of *Annonaceous* genotypes**

Primer	Sequence (5'- 3')	Primer	Sequence (5'- 3')
OPA – 02	GTGATCGCAG	OPH – 03	TGTCTGGGTG
OPA – 06	CAATCGCCGT	OPH – 05	AAAGCTGCGG
OPA – 10	TCGGCGATAG	OPH – 11	ACGCGCATGT
OPA – 13	CAGCACCCAC	OPH – 13	GACGCCACAC
OPA – 16	AGCCAGCGAA	OPH – 14	ACCAGGTTGG
		OPH – 15	AATGGCGCAG
		OPH – 16	TCTCAGCTGG
		OPH – 17	CACTCTCCTC
		OPH – 18	GAATCGGCCA
		OPH – 19	CTGACCAGCC

This is a fundamental step in the process of amplification of DNA by PCR, since the presence of polyphenols, polysaccharides, proteins, tannins, pigments, among others; are elements that damage the DNA, its precipitation and as a consequence, compromise subsequent molecular analyzes (9, 16).

The absence of DNA could also be due to the fact that in this kit no columns are used, so that the exposure time of the plant material and its manipulation was greater. Although the kits facilitate the isolation of genomic DNA and decrease the time of manipulation, it has been demonstrated in *Tectona grandis* L. that the lack of columns in these allows better results (17), compared to the classic methods with CTAB (18, 19, 20), employed in plants, although they do not surpass the quality that is obtained with those that if they have incorporated columns.

On the other hand, a new DNA isolation was performed by QIAGEN's DNeasy® extraction kit, which achieved excellent results (Figure 3), since it was possible to obtain genomic DNA of quality, purity and homogeneity. This can largely be due to the facilities and quality that allow the use of columns. In spite of the high costs of these DNA extraction kits, the great advantages, in terms of obtaining a quality DNA, the reduction of the manipulation and the smaller number of equipment used, have been demonstrated in specimens of the family *Juncaceae* (21).

The columns of the DNeasy® kit used in this study have, according to QIAGEN<sup>D</sup>, the anion exchange. It is argued that this property allows it to have a high affinity for nucleic acids, together with the use of chaotropic salts that break hydrogen bridges, increasing the solubility of non-polar substances in water, which fundamentally affect the secondary structure of polymers such as DNA, RNA and proteins (17).

DNA quality and homogeneity among samples are fundamental conditions for amplification (22). On the other hand, it is argued that the quality and purity of nucleic acids are two of the most important elements in molecular analyzes and cloning<sup>E</sup>. Another criterion is that having fast, reliable and low-cost protocols for extraction DNA is always desirable (9).

### DNA AMPLIFICATION WITH TYPE RAPD MOLECULAR MARKER

Of the three genomic DNA concentrations used (1, 2, 4 ng  $\mu\text{L}^{-1}$ ) with the three genotypes selected to establish the most suitable modifications, the best results were found when using 2 ng  $\mu\text{L}^{-1}$  and acceptable with 1 ng  $\mu\text{L}^{-1}$ . With this first, a greater number of bands were reached (Figure 4).

<sup>D</sup> QIAGEN. *QIAGEN® Product Guide Supplement* [en línea], 2011, [Consultado: 28 marzo 2015], Disponible en: [http://www.google.com/cu/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CB4QFjAA&url=http%3A%2F%2Fwww.qiagen.com%2Fresources%2Fdownload.aspx%3Fid%3Dc6c86754-4ffb-49ef-b927-a5773d28d6e9%26lang%3Den&ei=UucWVenaHI\\_2yQTIj4CQDw&usg=AFQjCNFMYy7QNGO-JPBtLctm5Lot5ddHLBg&bvm=bv.89381419,d.aWw&cad=rja](http://www.google.com/cu/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CB4QFjAA&url=http%3A%2F%2Fwww.qiagen.com%2Fresources%2Fdownload.aspx%3Fid%3Dc6c86754-4ffb-49ef-b927-a5773d28d6e9%26lang%3Den&ei=UucWVenaHI_2yQTIj4CQDw&usg=AFQjCNFMYy7QNGO-JPBtLctm5Lot5ddHLBg&bvm=bv.89381419,d.aWw&cad=rja).

<sup>E</sup> Somma, M. *Extracción y Purificación de ADN* [en línea], edit. Organización mundial de la salud Oficina Regional para Europa, 2011, Disponible en: <http://www.google.com/cu/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CB4QFjAA&url=http%3A%2F%2Fgmo-crl.jrc.ec.europa.eu%2Fcapacitybuilding%2Fmanuals%2FManual%2520ES%2FSesi%25C3%25B3n4.pdf&ei=-OgWVcvoHYazyASKvoKYBA&usg=AFQjCNGR15n6i7WigQ41a05Spguu64H4GA&bvm=bv.89381419,d.aWw&cad=rja>.

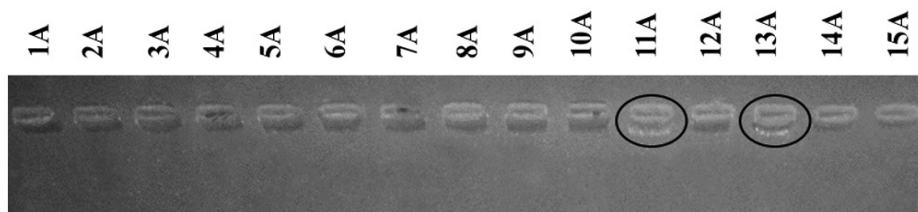


Figure 2. Results of 0.8% agarose gel electrophoresis from DNA extraction using the NucleonPHYTOpure Kit from selected *Anonaceae* accessions

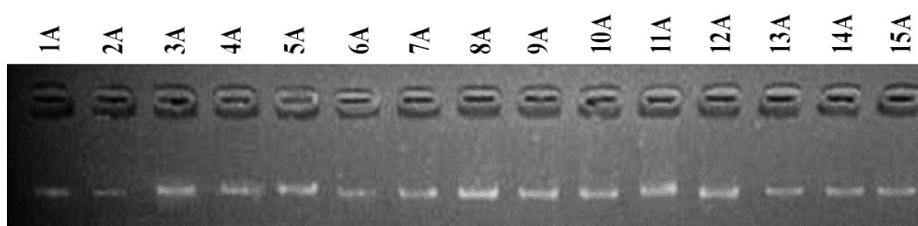


Figure 3. Results of 0,8 % agarose gel electrophoresis from DNA extraction by QIAGEN's DNeasy® Kit from selected *Anonaceae* accessions

As shown in the previous figure, with the three concentrations of genomic DNA used no amplification products (bands) were generated in the amplifications of *A. squamosa*, for this reason, it was decided to eliminate them from the other analyzes. One of the causes of the absence of bands is that the primers used did not find zones of DNA homology or genomic DNA quality in this species.

Although in the protocol optimization works with the molecular marker RAPD type, this is not one of the components that tend to vary, it is necessary to adjust the concentration to use because it affects the amplifications. The most recommended DNA concentration ranges to be used should be between 50-100 ng, since above this value amplification is affected by the dilution of the primers and below that sufficient amplification product is not obtained (23).

Another component that is varied in the amplification mixture is the concentration of the primer. Among the three concentrations used in the reaction (1, 2 and 3 ng  $\mu\text{L}^{-1}$  reaction) the best was the last one (Figure 5). When applying 1 ng  $\mu\text{L}^{-1}$  primer, virtually no amplification product was obtained, only one band in *A. reticulata*; Although with 2 ng  $\mu\text{L}^{-1}$  there is an increase of amplification products this is lower than that obtained with 3 ng  $\mu\text{L}^{-1}$ , in which the band pattern is more characteristic of a RAPD marker.

There is no established primer concentration for amplifications; this is an element that fits into each species and under different working conditions (9). These same authors, but in the species *Ceratozamia mexicana* Brongn., used primer doses of 1, 2 and 3 ng  $\mu\text{L}^{-1}$ ; however, with the former it is sufficient to obtain clear, distinctive and reproducible bands.

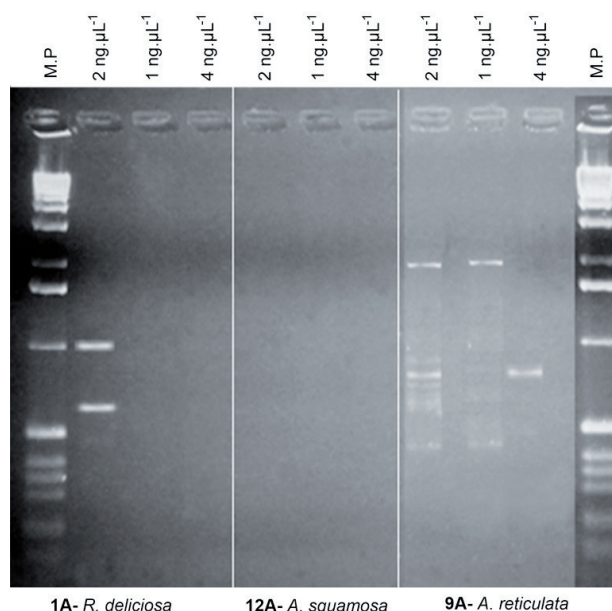


Figure 4. Amplification products obtained with different concentrations of genomic DNA (1, 2, 4 ng  $\mu\text{L}^{-1}$ ) in the genotypes used in the optimization of the RAPD protocol

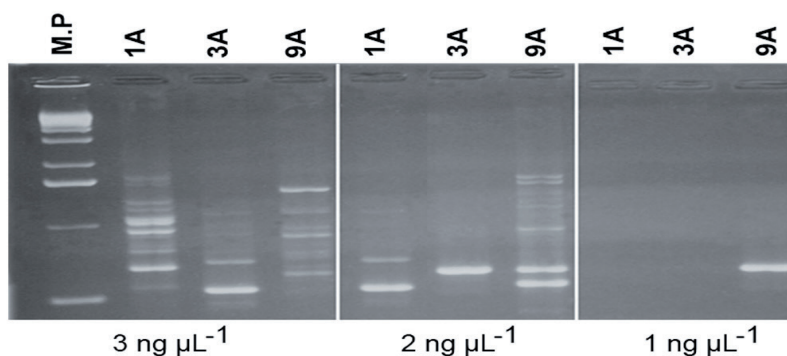


Figure 5. Amplification products obtained with the different concentrations of the primer (1, 2 and 3 ng  $\mu\text{L}^{-1}$  of reaction) used in the optimization of the RAPD protocol

DNA from the three selected accessions for screening amplified with all 15 primers tested. The amplification products of these three species revealed in the gels for each one, between two and five polymorphic bands (data not shown), which was corroborated in both replicates. Finally the four most polymorphic primers were selected: OPA-16, OPH-03, OPH-13 and OPH-18, from which the genetic diversity of the collection could be studied.

## CONCLUSIONS

- ◆ QIAGEN's DNeasy® extraction kit allows quality and purity DNA isolation from the accessions of the *ex situ* collection of Annonaceae from Cuba.
- ◆ The most effective concentrations in RAPD amplifications in Annonaceae are 2 ng  $\mu\text{L}^{-1}$  DNA and 3 ng  $\mu\text{L}^{-1}$  of the primer.

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### Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial