Journal of the Professional Association for Cactus Development

Optimization of DNA Extraction for ITS/U4U3 analysis of *Rhipsalis baccifera*

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Abstract. Genetic analysis of plants relies on high yields of pure DNA. For *Rhipsalis baccifera* this represents a great challenge since its plant tissue can accumulate large amounts of mucilage, polysaccharides, polyphenols and secondary metabolites, which co-purify with amplifiable DNA. These contaminating compounds lead to a poor yield and prevent access to PCR-based analysis. A number of factors, including choice of plant tissue, tissue preparation, and modifications of the extraction buffer, can impact on DNA extraction process. In this study, four different DNA extraction procedures were tested aiming to develop a simple protocol based on CTAB buffer. The results showed that the use of the outer cuticle of old lyophilized tissue allowed reliable results in *R. baccifera* plants with a good purity ranging from 1.6 to 1.8 and high DNA yield ≥ 500 ng μL^{-1}.

Key words: Cactaceae, mistletoe cactus, CTAB buffer, DNA yield, molecular markers

Cite: Rivas-García, T., Córdova-Pérez, D., Arredondo-Espinoza, R.C., Murillo-Amador, B., González-Estrada, R.R., Reyes-Pérez, J.J., Torres-Rodriguez, J.A., Martínez-Camacho, R.A. and Alejandre-Rosas, J.A. 2024. Optimization of DNA Extraction for ITS/U4U3 analysis of *Rhipsalis baccifera*. *Journal of the Professional Association for Cactus Development*. *26*:107-118. https://doi.org/10.56890/jpacd.v26i.563

Associate Editor: Pablo Preciado-Rangel.

Technical Editor: Benjamin Hernández-Vázquez.

Received date: 08 June 2024

Accepted date: 01 July 2024

Published date: 03 July 2024

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Introduction

One of the most noticeable and varied groups of angiosperms in warm dry America is Cactaceae family (Barrios et al., 2020). This family is monophyletic and is a member of the Caryophyllales order's suborder Portulacineae (Guerrero *et al*., 2019). The genus Talinum, which is the corresponding group to a subclade made composed of the genera Portulaca, Cactaceae, and Anacampseroteae, is at the base of this clade (de Araujo *et al*., 2021). Nonetheless, it is not clear the relationship of this clade members (Ocampo and Columbus, 2012). Except for the epiphytic *Rhipsalis baccifera* (J.S. Muell.) Stearn, which is found naturally in both the New and Old Worlds, the family Cactaceae is nearly exclusively found in the New World (Mauseth, 2016).

Rhipsalis baccifera (J.S.Muell) Stearn, commonly known as the mistletoe cactus, belongs to the genus *Rhipsalis* tribe *Rhipsalideae* of the subfamily Cactoideae (Barthlott *et al*., 1995). Due to its distributed areoles with tiny, bristly spine-like features (as opposed to massive, sclerified spines) and pendulous epiphytic habitat in the wet tropics, this species differs morphologically from its presumed terrestrial Cactoideae progenitors (Cota-Sánchez and Bomfim-Patricio, 2010).

It has caught the interest of numerous scientists since it is the only species of cactus that is found natively outside of the New World (Mauseth, 2016). It is still unknown, therefore, why *R. baccifera* is the only species in the Cactaceae family to natively occur outside of the New World, despite several suggestions having been put out to explain the species' distribution (Korotkova *et al*., 2011).

The six subspecies include *R. baccifera* subsp. *baccifera*, *R. baccifera* subsp. *mauritiana* (De Candolle) Barthlott, *R. baccifera* subsp. *erythrocarpa* (K. Schumann) Barthlott, *R. baccifera* subsp. *horrida* (Baker) Barthlott, *R. baccifera* subsp. *hileiabaiana* (J.L. Hage and H.S. Brito) N.P. Taylor and Barthlott and *R. baccifera* subsp. *shaferi* (Britton and Rose) Barthlott and N.P. Taylor (Barthlott *et al*., 1995). Furthermore, *R. baccifera* has experienced multiple polyploidization events, with chromosome numbers ranging from diploid ($2n = 2x = 22$) to tetraploid ($2n = 4x = 44$) to octaploid ($2n = 8x = 88$) (Barthlott *et al*., 1995). Higher levels of polyploidy are associated with greater geographic distance from Brazil, which is the center of variety (Christenhusz and Chase, 2012).

It has medicinal use since its stem is crushed and combined with juice of *Lonchocarpus chrysophyllus* Kleinhoonte to treat coral snake bites (*Micrurus* sp.). It is also used with *Philodendron* sp. to relieve venomous stingray (*Potamotrygon* sp.) wounds (Bautista-San Juan *et al*., 2017). Additionally, ethanolic extracts of *R. baccifera* have antitumor activity of up to 84.1 and 75.8% at concentrations of 250 mg kg^{-1} and 125 mg kg^{-1}, respectively, also having low acute toxicity (Machado and de Oliveira, 2020). Previous studies of *R. baccifera* are encouraged to understand its distribution, molecular systematics and morphometric characters (Barthlott *et al*., 1995; Korotkova *et al*., 2011; Oulo *et al*., 2020).

Nonetheless, there are many gaps in its molecular research. It is therefore essential to characterize, morphometric and genetically, the different populations of *R. baccifera.* This could be related to the difficulty to isolate high-quality DNA from *R. baccifera* tissues, a key element in such studies that use many molecular techniques. The difficulties encountered while working with this species are caused by the presence of high amounts of mucilage, polysaccharides, polyphenols and secondary metabolites (Asri and Hanafi, 2023). In addition, these contaminants interfere in downstream reactions such as DNA restriction, amplification and cloning. The objective of this study was to optimize an extraction protocol with an attempt to isolate high-quality DNA from *R. baccifera* plant. ITS/U4U3 amplification was also performed to evaluate the suitability of the extracted DNA for PCR-based techniques.

Materials and Methods

Plant material

In August of 2023, fresh and healthy plant tissue of *R. baccifera* were randomly sampled from 10 individuals of different geographically representative natural populations in Veracruz (Orizaba, Campo chico, and Ixtaczoquitlán), Mexico (Table 1). The choice of tissue for DNA extraction from the plant mucilaginous is a critical step.

In this study, different types of plant tissue were tested (Data not shown). For the above mentioned, a portion from the outer cuticle was carefully cut and peeled, using a sterilized scalpel, to remove the internal mucilage. After collection, the ten sampled plants were ground in a mortar with pestle and liquid nitrogen or lyophilized (Table 1) and stored at -80°C until DNA extraction, and further molecular marker analysis.

Table 1. Location of ten plant tissues of *R. baccifera* sampled randomly from different geographically populations in Veracruz.

ID Sample	Location
1	18°49’60’’N, 96°59’14’’W
2	18°50’10’’N, 69°59’50’’W
3	18°50’17’’N, 96°59’48’’W
4	18°49’33’’N, 97°00’28’’W
5	18°51’38.08’’N, 97°1’49.91’’O
6	18°51’38.08’’N, 97°1’49’’O
7	18°51’38.08’’N, 97°1’50’’O
8	18°51’39.69’’N, 97°01’47.97’’O
9	18°51’39’’N, 97°01’48’’O
10	18°49’’58.28’’N, 97°05’01.63’’O

Reagent and solutions

The extraction buffer consisted of 80 mM CTAB (Sigma, Mexico), 100 mM Tris-HCl pH 8.0 (CalBiochem, USA), 20 mM EDTA pH 8.0 (Merck, USA). After being autoclaved for 20 min, 0.75 mM PVP (mol wt 40.000; Sigma, Sintra, Mexico), and 28 mM 2-mercaptoethanol (Merck, Mexico) were added to the extraction buffer, immediately before use. In addition, chloroform: isoamyl alcohol (24:1, v/v, Sigma, Mexico), 70% (v/v) ethanol (Merck, USA), phenol (1:1, v/v, Sigma, Mexico), proteinase K (20 mg mL^{-1}, Sigma, Mexico), ribonuclease A (10 mg mL^{-1}, RNase-A, Sigma, Mexico), and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA. Absolute iso-propanol (Merck, USA), and absolute ethanol (Sigma, Mexico) were also required.

DNA extraction

The commonly used DNA extraction method, developed by Doyle and Doyle (1987), using cetyl trimethyl ammonium bromide (CTAB) in the extraction buffer was tried in the beginning. Since results proved to be unsatisfactory, three modified CTAB protocols (method A, B, and C) (Table 2) were developed and tested and the commercial Genomic DNeasy Plant mini kit (QIAGEN).

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Table 2. DNA extraction CTAB protocols tested and important modifications to standard procedure.

Modified steps of CTAB protocol	Method A	Method B	Method C
Mechanical digestion	Liquid nitrogen	Liquid nitrogen	Lyophilized tissue
Chemical digestion	Extraction buffer: CTAB 80 mM, NaCl 1.4 M, EDTA 20 mM, Tris-HCl 100 mM, PVP 0.75 mM, 2-mercaptoetanol 28 mM, 13.7 M H_2O.	Extraction buffer: CTAB 80 mM, NaCl 1.4 M, EDTA 20 mM, Tris-HCl 100 mM, PVP 0.75 mM, 2-mercaptoetanol 28 mM, 13.7 M H_2O. RNase A (10 mg mL^{-1}), Incubation for 30 min at 37°C.	Extraction buffer: CTAB 80 mM, NaCl 1.4 M, EDTA 20 mM, Tris-HCl 100 mM, PVP 0.75 mM, 2-mercaptoetanol 28 mM, 13.7 M H_2O. Proteinase K (20 mg mL^{-1}), Incubation for 30 min at 37°C.
Elution	Washing two times with chloroform: isoamyl alcohol (24:1, v/v). Then washing with absolute iso-propanol. Then washing with 70% (v/v) ethanol. 60 µL of deionized water	Washing with phenol (1:1, v/v). Then washing overnight with 2X absolute ethanol. Then washing with 70% (v/v) ethanol. 60 µL of deionized water	Washing with 2% (v/v) 2-mercaptoethanol. Washing two times with chloroform: isoamyl alcohol (24:1, v/v). Then washing with 70% (v/v) ethanol. 50 µL TE buffer

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DNA extraction protocol

The optimized procedure, which allowed the great improvement on both yield and purity, was described as follows. Plant tissues of *R. baccifera* (200 mg of lyophilized) were transferred to a 2 mL micro tube containing 1.3 mL of pre-heated (70 °C) extraction buffer. The tube was mixed by inversion and left to stand for 2 min at room temperature. After that, 10 µL of proteinase K (20 mg mL^{-1}) was added and the mixture was mixed again by inversion for 1 min. The mixture was incubated at 65 °C in a water bath for 1 h with occasional mixing. Then, 5 µL of 28 mM 2-mercaptoethanol was added, and incubated at 65 °C in a water bath for 15 min with occasional mixing. Then, the tube was centrifuged at 14,000 rpm, for 1 h at 4 °C and the supernatant was transferred to a clean 2 mL micro tube. After that, 6 mL of chloroform: isoamyl alcohol (25:24:1) was added, mixed by using gentle inversion for 5 min, incubated on ice for 10 min and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was transferred to a clean 2 mL micro tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added. After two times of chloroform: isoamyl alcohol (24:1) addition, and the second centrifugation with volume transfer, 6 mL of absolute iso-propanol was added and incubated at 20 °C for 1 h. The tube was then gently inverted for 5 min, incubated on ice for 10 min and centrifuged at 10,000 rpm for min at 4 °C. The upper aqueous phase was transferred to a clean 1.5 mL micro tube and DNA was precipitated by adding 5 mL of ice-cold 70% ethanol and centrifuged again at 13,000 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was air-dry for 20 min at room temperature. Finally, the pellet was re-suspended in 60 µL of TE buffer and stored at −20 °C.

DNA quantification and quality assessment

The quantity and quality of the DNA obtained was assessed spectrophotometrically at 260 and 280 nm, and the A_{260}/A_{280} ratio was used to assess contamination with proteins. This spectrophotometric analysis was performed in triplicate on the samples of extracted DNA, in a PG Instruments Ltd. T70 UV/VIS spectrometer. In order to verify its integrity, 3 µL DNA were subjected to gel electrophoresis on 1.8% (w/v) agarose gel, stained with ethidium bromide (0.5 µg mL^{-1}) (Sambrook *et al*., 1989). The DNA migration was performed in 1X TBE running buffer (Tris, boric acid, EDTA) at voltage of 100 V for 120 min. A 1,000 bp molecular weight standard (Invitrogen 1 Kb Plus DNA Ladder) was used. The agarose gel was visualized under UV transilluminator and photo-documented with the aid of the Gel Logic 112Pro imaging system (Carestream, Rochester, NY).

ITS/U4U3 amplification

The nuclear ribosomal intergenic spacer (ITS) was used to test quality and performance of the DNA extracted from method C, which proved to be the most efficacious compared to others methods tested in the present study (see results). The DNA amplification was carried out from the 5.8s ribosomal gene with the primers F-ITS-U4 (5'-RGTTTCTTTTCCTCCGTTA-3') and R-ITS-U3 (5'-CAWCGATGAAGAACGYAGC-3'). To carry it out, 20 µL of master mix was used (5 µL of Green Flexi Reaction Buffer 5x), 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of DNTPs, 1.2 µL of ITS U4 primer at 8.3 µM and 1.2 µL of ITS U3 primer at 8.3 µM, 0.2 µL of Taq Pol (1 unit per reaction) and 10.4 µL of deionized water) and 5 µL of DNA sample (10 ng μL^{-1}). Reactions without DNA were used as negative controls.

For the amplification of the DNA samples, a thermocycler (NYX Technik Apollo ATC 201) was used and the polymerase chain reaction (PCR) was based on the following conditions, 35 cycles, initial denaturation at 94 °C for 4 min, followed by an extension at 94 °C for 30 s, alignment at 60 °C for 50 s, elongation at 72°C for one minute and a final extension at 72 °C for 10 min. Subsequently, 3 µL of amplicons were subjected to gel electrophoresis on 1.8% (w/v) agarose gel, stained with ethidium

bromide (0.5 μg mL^{-1}) (Sambrook *et al.*, 1989). The DNA migration was performed in 1X TBE running buffer (Tris, boric acid, EDTA) at voltage of 100 V for 120 min. A 1,000 bp molecular weight standard (Invitrogen 1 Kb Plus DNA Ladder) was used. The agarose gel was visualized under UV transilluminator and photo-documented with the aid of the Gel Logic 112Pro imaging system (Carestream, Rochester, NY).

Data analysis

The normality of the data was determined using the Shapiro-Wilk test and the homogeneity of variances using the Bartlett test. The data were analyzed by one-way analysis of variance (ANOVA) using STATISTICA 10.0 software (StatSoft, 2011) and Fisher's mean multiple comparison test was used ($P < 0.05$).

Results and discussion

The isolation of the outer cuticle is difficult from young and fresh cladodes because of the interference from the mucilage. The mucilage is a compound that is found naturally in the large cells of chlorenchyma and nearby water-retentive parenchymal cells (Asri and Hanafi, 2023). Besides, fresh tissue from cacti contains many polyphenolics and polysaccharides that coprecipitate with DNA and affect subsequently the PCR amplification (Shedbalkar *et al*., 2010). Thus, Mondragon-Jacobo *et al*. (2000) involved a DNA extraction method for several cacti species (*e.g. Cleistocactus* spp., *Echinocereus* spp., *Nopalea* spp., *Opuntia* spp., and *Stenocereus* spp.), and they concluded that the amount of tissue used depends on the specie and other factors such as mucilage content, age of sampled tissue, and sample size. As reported by Griffith and Porter (2003) who extracted DNA from epidermal cells from several species including Opuntia, this study confirms that the use of the outside of cuticle of old tissue samples minimizes the mucilage content in *R. baccifera* plants.

In the present study, one commercial kit and three improved CTAB methods (method A, B and C) for DNA extraction were applied to *R. baccifera* DNA. The protocol of Doyle and Doyle (1987) is widely used as a standard guideline to conduct CTAB-based DNA extraction methods in various studies (Sing and Kumar, 2012). Firstly, the CTAB method, which proved to be inadequate was tested. With this method no DNA was extracted (Table 3). As mentioned before, this is probably due to the specific characteristics of this plant, like the presence of pectin, mucilage, polysaccharides, alkaloids, phenolic, and terpenes, which either lead to embedding of DNA into a sticky gelatinous matrix (Do and Adams, 1991) or promote DNA degradation (Wink, 2015). In this study, the Doyle and Doyle-CTAB protocol was modified and tested a commercial kit to improve DNA yield and quality.

Table 3. Yield and purity of DNA extracted from the outer cuticle of *R. baccifera* by four different methods.

Methods	DNA yield (ng μL^{-1})	DNA purity (A_{260}/A_{280})	DNA purity (A_{260}/A_{230})
DNA kit	1,010.5 ± 8 b*	1.53 ± 7 b	1.6 ± 3 c
CTAB-A	840.40 ± 15 *c	1.34 ± 6 c	1.1 ± 6 d
CTAB-B	1,014.35 ± 21 b	1.61 ± 11 b	1.8 ± 2 b
CTAB-C	2,012.22 ± 11 a	1.83 ± 16 a	2.1 ± 4 a

*Means with the same letters in the columns do not differ significantly according to Fisher test ($P \leq 0.05$). ± Standard deviation.

The CTAB-based DNA extraction and commercial kit DNA extraction could be differentiated using their basis and format (Asri and Hanafi, 2023). The CTAB-based is a chemical solution and enzymatic lysis

based which requires DNA to precipitate in a chemical solution and enzymes for about 30 to 45 minutes, are less costly, allow for both large and small samples to be applied and have higher yields. Meanwhile, for commercial DNA extraction kits such as Genomic DNeasy Plant mini kit (QIAGEN), Vivantis GF-1 nucleic extraction kit, Nucleospin plant II kit-lysis buffer PL1 and PL2 is silica-membrane based which requires DNA binds selectively to the silica matrices, produce more rapid results than CTAB-DNA based methods. Nonetheless, those methods are more expensive, and have slightly lower yield and large sample limitation (Schiebelhut *et al.*, 2017).

The good quality of the DNA was assumed by the A_{260}/A_{280} absorbance ratio. Based on composition of DNA, the absorbance at a wavelength of 260 nm is directly proportional to DNA concentration and absorbance readings at 280 nm measure the concentration of proteins. In general, since of the presence of salts, carbohydrates and other contaminants in the samples, the absorbance readings at 230 nm wavelengths must be considered. So the typical values of the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios are the range of 1.6-1.9 and 2.0-2.2, respectively (Sambrook *et al.*, 1989). Using the protocols described above, the absorbance analysis for all samples tested were satisfactory with method B and C for the ratio A_{260}/A_{280} and C for the ratio A_{260}/A_{230}, respectively. On the other hand, the values obtained by method A and DNA kit were unsuitable for desired DNA purity values. The method C produces higher yield compared with the other methods tested, however all protocols generate a high DNA yield ≥ 500 ng μL^{-1}.

Although DNA yield was incremented in method from DNA kit and method B, another problem persists by the presence of contamination compounds in the DNA samples, particularly proteins and polysaccharides, as visualized in the agarose gel (Fig. 1) and confirmed by the low ratios obtained (Table 3).

Based on the results from the gel electrophoresis (Fig. 1), the method CTAB A and DNA kit are the less recommended for DNA extraction from *R. baccifera* species. This is due to the non-visualized DNA extraction and low quality of DNA extracted compared to the others tested methods. During DNA extraction of cacti, pectin and polysaccharides are the most difficult contaminants to separate from DNA (Martínez-González *et al.*, 2017). The pectin becomes the primary constituent of the cellular wall in cacti taxa, and frequently varies depending on the species, the location, and the surrounding environment (Picot-Allain *et al.*, 2022). Like other plant species, cacti have secondary metabolites and polysaccharides that block enzyme actions (Raina and Chandlee, 1996). The presence of polysaccharides is visible due to their viscous, glue-like texture, which makes it difficult to pipette the DNA and makes it even more difficult for the PCR to work (Porebski *et al.*, 1997; Schrader *et al.*, 2012).

The suitable of isolated DNA from the optimized protocol (method C) in molecular techniques were assessed by ITS/U4U3 analyses, which is useful to evaluate the genetic diversity and phylogenetic relationship (Bezerra *et al.*, 2022). The results show that the DNA extracted from the optimized protocol was of suitable quality to screen levels of genetic diversity and phylogenetic relationship and proving that the DNA can be amplified via PCR (Fig. 2).

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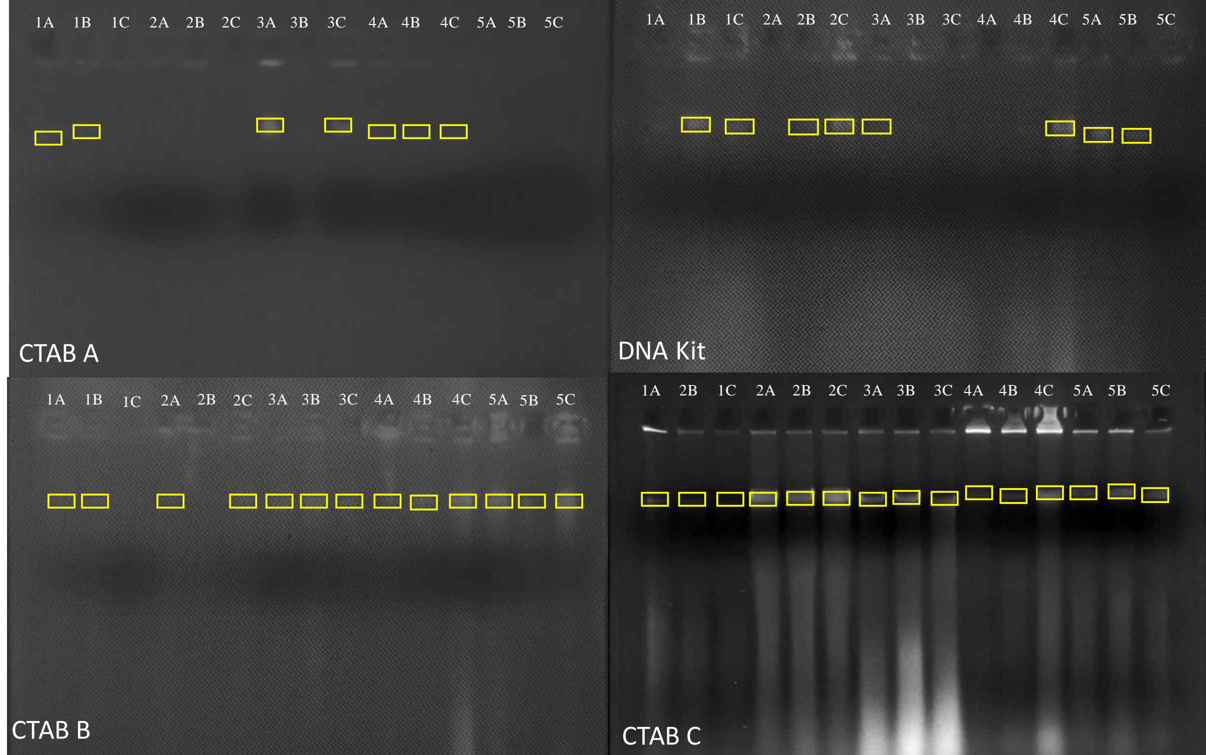

Figure 1. Electrophoretic pattern of DNA extracted by three modified CTAB protocols (method A, B, and C), and the commercial Genomic DNeasy Plant mini kit (QIAGEN) from the outer cuticle of *R. baccifera*. The electrophoresis was performed in 1.8% agarose gel (w/v).

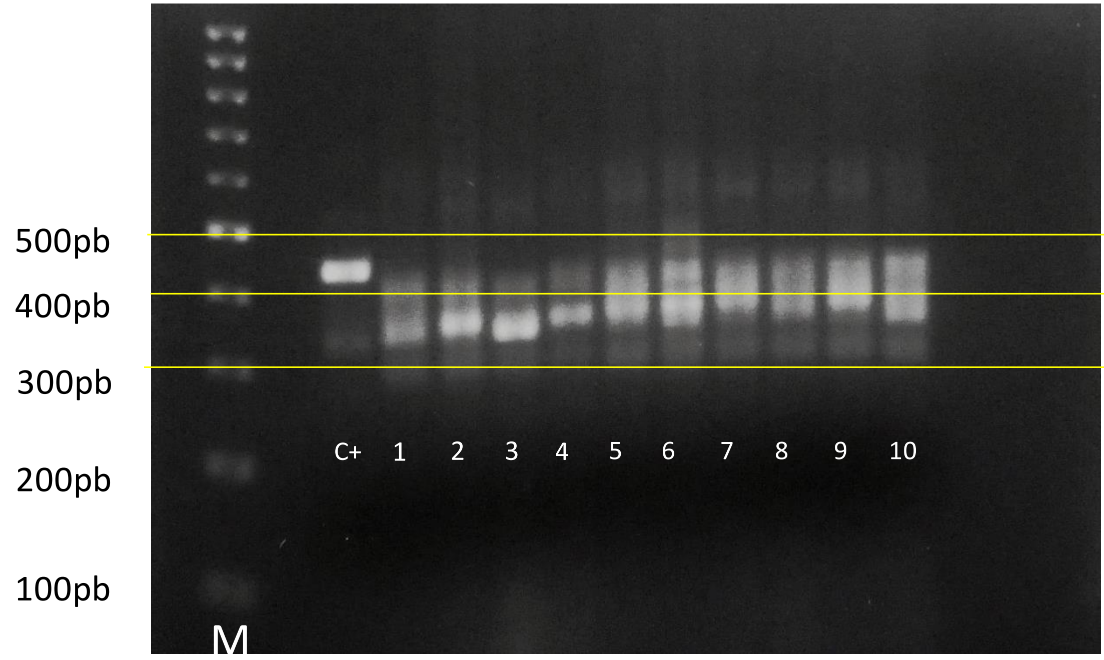

Figure 2. ITS/U4U3 Amplification of DNA from 10 *R. baccifera* individuals. M: Molecular marker of 1,000 bp molecular weight standard (Invitrogen 1 Kb Plus DNA Ladder). The electrophoresis was performed in 1.8% agarose gel (w/v).

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For a detailed analysis of diversity, sequencing is used, therefore good amplification will facilitate the reading of the chains to be sequenced, which is why having the least amount of errors that affect, such as obtaining false mutations that prevent and delay sequencing (Potapov and Ong, 2017). The most common errors during amplification occur during preparation, such as the use of poor quality DNA, poor incorporation of the polymerase, and it is also possible to find errors due to template change and recombination mediated by PCR (Vijg, 2021). Complete removal of polysaccharides during DNA isolation assumes critical importance due to their well-established interference problems, namely failure of DNA amplifications during PCR due to inhibition of *Taq* polymerase activity (Fang *et al*., 1992).

Conclusions

In this study, a method of DNA extraction that yields high-quality DNA free of inhibitory organic compounds common in plants of cacti, such as pectin and mucilage was developed. The advantage is that only a small amount of tissue is required for DNA extraction, it does not require sophisticated equipment, and it is cheaper than the commercial kits for DNA extraction. This improved method was quick and simple to be conducted without the need for further purification. Also, this method allowed to obtain excellent and adequate quality of DNA that can be used in further PCR-based molecular marker´s analyses.

ETHICS STATEMENT

Not applicable

CONSENT FOR PUBLICATION

Not applicable

COMPETING INTEREST

The authors declare that they have no competing interests.

AVAILABILITY OF SUPPORTING DATA

Not applicable

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

Conceptualization, R.C.A-E. and J.A.A-R; methodology, D.C-P; software, R.R.G-E.; validation, B.M-A, T.R-G. and J.J.R-P.; formal analysis, J.A.T-R.; investigation, D.C-P.; resources, T.R-G.; data curation, R.M-C.; writing—original draft preparation, J.A.A-R.; writing—review and editing, T.R-G..; visualization, R.R.G-E.; supervision, T.R-G.; project administration, J.J.R-P; funding acquisition, T.R-G. and J.A.A-R.

ACKNOWLEDGEMENTS

Not applicable

https://www.jpacd.org Electronic ISSN: 1938-6648

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