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African Journal of **Biotechnology**

7 November 2018
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

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Table of Content

Comparison of four DNA extraction methods in Heliconia (Heliconia L.)

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Avendaño-Arrazate C., Olivera A., Canul J. and Grajales M.

Full Length Research Paper

Comparison of four DNA extraction methods in *Heliconia* (*Heliconia* L.)

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Received 15 April, 2018; Accepted 29 October, 2018

Molecular analysis in plants requires the use of high quality DNA. Currently, there are a myriad of DNA extraction protocols, which still seems that their applicability across different species is not straightforward. The aim of the present study was to evaluate four different cetyl trimethylammonium bromide (CTAB)-based DNA extraction protocols for *Heliconia* species from lyophilized fully mature leaves. A split-plot design with two levels (six different species and four DNA extraction methods) was used, where treatments were distributed in a randomized block design with four replications. The response variables were total yield (μg) and purity (A_{260}/A_{280} nm), and were determined by UV-spectrophotometry and agarose gel electrophoresis, respectively. Additionally, *EcoRI* enzyme restriction digestion and amplification of the *Hc_D6* locus marker were used to evaluate the functionality of the extracted DNA. Analysis of variance (ANOVA) results indicated that the extraction methods had a significant effect on the DNA yield ($F = 8.51$, $df = 3$, $P < 0.0001$) and purity ($F = 10.43$, $df = 3$, $P < 0.0001$). The best methods to obtain DNA from *Heliconia* spp. were those described by Michiels et al. (2003) and Sagahi-Marooof et al. (1984) with modifications.

Key words: DNA extraction, *Heliconia*, tropical plants, cetyl trimethylammonium bromide (CTAB), polyvinylpyrrolidone (PVP).

INTRODUCTION

Current protocols in molecular biology require the use of high quality DNA. For some plant species, this rather common process can turn into a burdensome task, due to the presence of secondary metabolites. These compounds may undergo rapid oxidation, binding tightly to the DNA and subsequently, co-precipitate with the DNA preventing its use in downstream applications (Porebski et al., 1997; Sheperd et al., 2002; Shabnam

and Saeed, 2016; Swati et al., 2016). Tropical plants for example, often have high levels of polysaccharides, polyphenols, proteins, lipids, among others, which make the isolation and purification of DNA difficult (Barra et al., 2012; Cavallari et al., 2014; Huang et al., 2014). In addition, the presence of these compounds in the isolated DNA inhibits the activity of various enzymes used in molecular biology experiments, such as DNA

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Table 1. Reagents used in the extraction buffer for each method.

Method	Tris-HCl (mM)	NaCl (mM)	EDTA (mM)	CTAB (%)	BME (mM)	PVP (%)	Reference
1	100	700	50	3.5	420	3	Haque et al. (2008)
2	100	700	50	1.0	140	1	Saghai-Marroof et al. (1984)
3	100	700	50	2.0	280	-	Doyle and Doyle (1990)
4	100	700	50	2.0	280	2	Michiels et al. (2003)

ligases, polymerases and endonucleases, affecting their efficiency, and in many cases rendering negative results (Friar, 2005; Cavallari et al., 2014; Swati et al., 2016).

The research team of this study worked on the population genetics of the native heliconia species from the southern part of Mexico (Avendaño-Arrazate, 2017). Heliconias are tropical plants that have been cultivated as ornamentals outside their natural distribution areas, increasing their importance as cut flowers. The genus *Heliconia* L. displays a great array of diversity of species, varieties, hybrids and cultivars, often leading to taxonomic inaccuracies among scientists and commercial growers (Marouelli et al., 2010). The majority of the studies have focused mostly on taxonomy (Iles et al., 2017), regeneration and *in vitro* propagation and very few on morphological (Arrazate-Avendaño et al., 2017) and molecular characterization (Marouelli et al., 2010; Isaza et al., 2012). Therefore, more molecular studies are needed to clarify these discrepancies and the isolation of high quality DNA is one of the central steps to achieve it.

Although there are few reports about extracting DNA from these species, in some cases it requires the use of robotics (Côrtes et al., 2009), additional purification steps after the DNA extraction, using commercial kits (Meléndez-Ackerman et al., 2005) or the use of phenol-chloroform cleaning steps (Kumar et al., 1998; Sheela et al., 2006). Therefore, the objective of this study was to compare four commonly used cetyl trimethylammonium bromide (CTAB) DNA extraction methods with modifications: Haque et al. (2008) Method 1; Saghai-Marroof et al. (1998) Method 2; Doyle and Doyle (1990) Method 3 and Michiels et al. (2005) Method 4. The starting material was mature leaves and the effectiveness of the methods was tested based on yield, purity, integrity and functionality, in order to determine the most suitable method for isolating high yield of quality DNA from *Heliconia* spp.

MATERIALS AND METHODS

Plant

Fully developed leaves were collected from the "Rosario Izapa Experimental Field Station" (Chiapas, Mexico). Four different heliconia species were used: *Heliconia stricta* Hubert, *Heliconia wagneriana* Peterson, *Heliconia bourgaeana* and *Heliconia collinsiana* Griggs. Additionally, leaf samples from *Canna indica* L. species belonging to the Zingiberales order, were also included as

control. After harvest, leaf tissue was kept at -80°C for at least 48 h prior to lyophilization. Freeze-dried tissue was ground into fine powder using the TissueLyser II (Qiagen, Hilden, Germany).

Experimental design

A split-plot design with two levels (six different species and four DNA extraction methods) was used to determine the best extraction method for heliconias. The big plots were the DNA extraction methods, and the different species were the split-plots. Treatments were distributed in a randomized block design with four replications. Three samples per species in each method were extracted (12 samples of the same species per replicate). In total, 240 DNA extractions were carried out. The response variables were total yield (µg) and purity (A260/A280 nm). All DNA extractions were carried out following the same procedure to control the variation inherent to the different steps of each method. Statistical analyses were performed using SAS PROC GLM to test the effect of the extraction method on DNA yield and purity. Means were compared using the Tukey test with a significance level of 0.05 (SAS, 2011).

DNA extraction

All DNA extractions were carried out with 50 mg of lyophilized tissue in a 2.0 ml tube. Extraction buffer (1 ml) was added to each tube followed by incubation at 65°C for 45 min with constant mixing (Table 1). Tubes were allowed to cool at room temperature (RT) for about 5 min and to each tube, 700 µl of chloroform:isoamyl alcohol (24:1) were added. The tubes were inverted gently for 10 min to form an emulsion and were centrifuged at 10,000 × *g* for 10 min at RT. The upper phase was decanted into a new tube and the washing step was repeated. After the second centrifugation, about 600 µl were transferred to a new 2.0 ml tube containing 5 µl of RNaseA (10 mg/mL) and incubated at 37°C for 30 min. DNA was precipitated with 600 µl of cold isopropanol, mixing gently by inversion several times until DNA was visible.

The tubes were centrifuged at 10,000 × *g* for 10 min at RT and the supernatant was carefully poured off. The DNA pellets were washed twice with ethanol at 70 and 95%, for 5 min and were spun again as before. The DNA pellets were allowed to dry at RT and were resuspended in 300 µl of TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0) overnight.

Measurement of DNA quantity, purity and integrity

DNA concentration was measured using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The A260/A280 nm absorbance ratio was used to assess the purity of the nucleic acid samples (Held, 2001). Integrity of the extracted DNA was evaluated by electrophoresis of 100 ng/well for each sample on 1% agarose gel. Migration distance was compared to that of 100 ng of uncut lambda DNA (NEB, Ipswich, MA, USA).

Table 2. DNA yield (μg) and $\text{OD}_{A260/A280}$ ratio for DNA extractions from 50 mg of lyophilized leaf tissue from *H. stricta*, *H. wagneriana*, *H. bourgaeana*, *H. collinsiana*, and *C. indica* by four extraction methods.

Species	Method 1		Method 2		Method 3		Method 4	
	Yield	OD	Yield	OD	Yield	OD	Yield	OD
<i>H. stricta</i>	39.83±04.90	2.04±0.06	39.15±04.45	1.98±0.02	39.60±02.30	1.99±0.04	41.55±04.33	1.99±0.02
<i>H. wagneriana</i>	53.40±11.85	2.20±0.08	54.00±12.71	2.15±0.05	57.23±23.26	2.14±0.08	66.08±22.51	2.16±0.08
<i>H. bourgaeana</i>	63.00±08.23	1.97±0.00	71.63±12.94	1.91±0.05	50.18±12.56	1.93±0.03	78.00±11.37	1.93±0.02
<i>H. collinsiana</i>	36.00±04.22	2.10±0.12	61.28±07.37	2.04±0.13	45.23±06.11	1.95±0.04	49.73±08.97	1.98±0.07
<i>C. indica</i>	49.50±01.41	2.35±0.04	62.48±06.97	2.22±0.02	36.98±12.33	2.60±0.11	66.38±16.86	2.21±0.07
Mean	48.35±11.75	2.13±0.15	57.71±13.96	2.06±0.13	45.81±14.04	2.12±0.26	60.35±18.42	2.05±0.13

Restriction endonuclease digestion

To assess the functionality of the extracted DNA for downstream applications, 50 ng of DNA were digested with 15 units of *EcoRI* (NEB, Ipswich, MA, USA) in a 20 μl reaction for 5 h at 37°C. Digested DNA was assessed by visual inspection on a UV transilluminator (Kodak Gel Logic 112, Woodbridge, CT, USA) after 1% agarose gel electrophoresis and GelRed™ staining (Biotium, Hayward, CA, USA).

PCR amplification

DNA amplification reactions of the *H. caribaea* specific microsatellite marker (*Hc_D6*) were performed only for *Heliconia* spp. samples (*C. indica* was excluded since this marker is specific for *Heliconia*) in a 10 μl reaction (Gowda et al., 2012). The PCR cocktail consisted of 10 ng of DNA, 4 μl of 2X RedTaq® Ready Mix™ (SIGMA-ALDRICH, St. Louis, MO, USA), 0.5 μM of each primer (For 5'ACT GCA CAT CAT ATC ATC CTG3' and Rev 5'GTG GGT CAG TCA ATT ACT GTG3') and sterilized water. The expected size of the *Hc_D6* product was 222 to 258 bp. Amplifications were carried out on a PrimeG Thermal Cycler (Techne Inc., Burlington, NJ, USA). The amplification was carried out with an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C and a final extension step of 6 min at 72°C. PCR amplification products were visualized as described previously.

RESULTS AND DISCUSSION

DNA yield

Yield of the extracted DNA by the four different methods is shown in Table 2. The extraction methods had a significant effect on the DNA yield ($F = 8.51$, $df = 3$, $P < 0.0001$). In general, the extraction methods that contain polyvinylpyrrolidone (PVP, MW 40,000, Sigma-Aldrich, St. Louis, MO, USA), had significantly higher DNA yields. Methods 4 and 2 (Michiels et al., 2003; Saghai-Marouf et al., 1984) were statistically superior for extracting DNA in the evaluated species. Each of these methods yielded on average 60.35±18.42 and 57.70±13.96 μg , compared to Method 3, with 45.84±14.04 μg , which had the lowest yield (Figure 1). Methods 2 and 4 included PVP in their extraction buffer while Method 3 did not. PVP has been reported to form insoluble complexes with lactones and phenolic compounds (Kim et al., 1997; Barra et al., 2012), and the presence of these compounds in *Heliconia* spp. have been detected in different studies (Estrada et al., 2008; Hernández-Meneses et al., 2013). Additionally, through the centrifugation steps after each the addition of

chloroform: isoamyl alcohol, these compounds are removed by precipitation (Haque et al., 2008; Swati et al., 2016; Abdel-Latif and Osman, 2017). These steps, in combination with the use of CTAB, aided the selective precipitation of DNA, which could explain the higher concentrations of DNA obtained by these methods.

DNA purity and integrity

The purity of the DNA obtained was evaluated as a function of the $\text{OD}_{A260/A280}$ ratio (Held, 2001). The mean $\text{OD}_{A260/A280}$ ratios for the four methods evaluated in this research ranged from 2.05 to 2.13 (Table 2). The statistical analysis indicates that the extraction methods had a significant effect on the DNA purity ($F = 10.43$, $df = 3$, $P < 0.0001$), where Methods 2 and 4 had the narrowest range for the $\text{OD}_{A260/A280}$ ratio (Figure 1). Method 3 had the highest $\text{OD}_{A260/A280}$ ratio values (2.76, outlier value in Figure 1); however, all these values came from the *C. indica* samples (which had a similar trend regardless of the method). In general, Methods 1, 2 and 4 had $\text{OD}_{A260/A280}$ ratio values within the commonly accepted range (1.8 to 2.0). These values can be explained by the removal of polysaccharides and proteins, usually

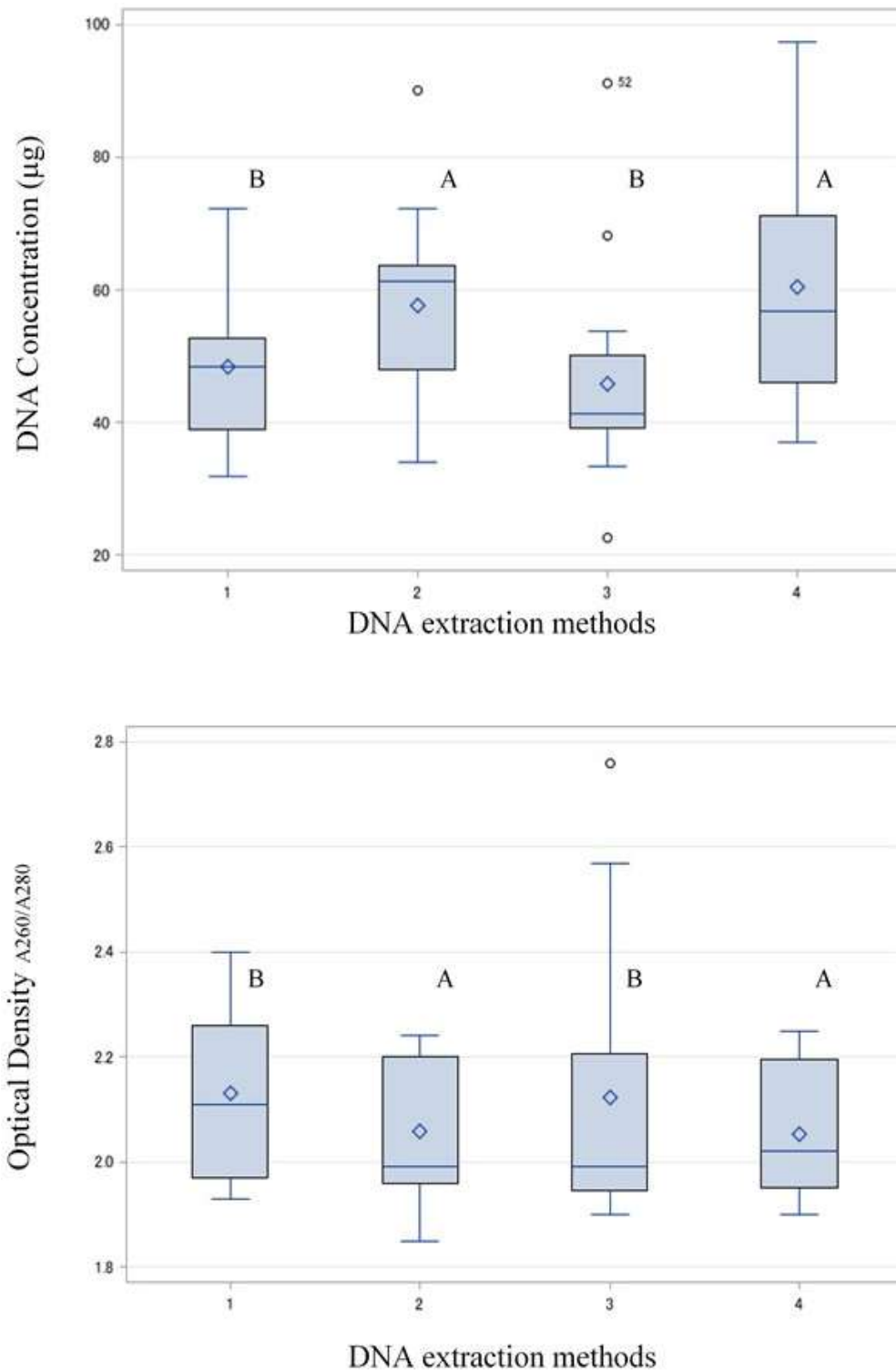


Figure 1. Box plot diagram of the variable yield (upper figure) and DNA quality (lower figure) obtained for each extraction method. Different capital letters indicate significant differences ($P < 0.001$) among the evaluated methods according to the Tukey's test.

bound to the DNA. With the addition of NaCl in the extraction buffer, the solubility of these compounds is

increased and the use of the chloroform: isoamyl alcohol wash steps reduces their co-precipitation with the

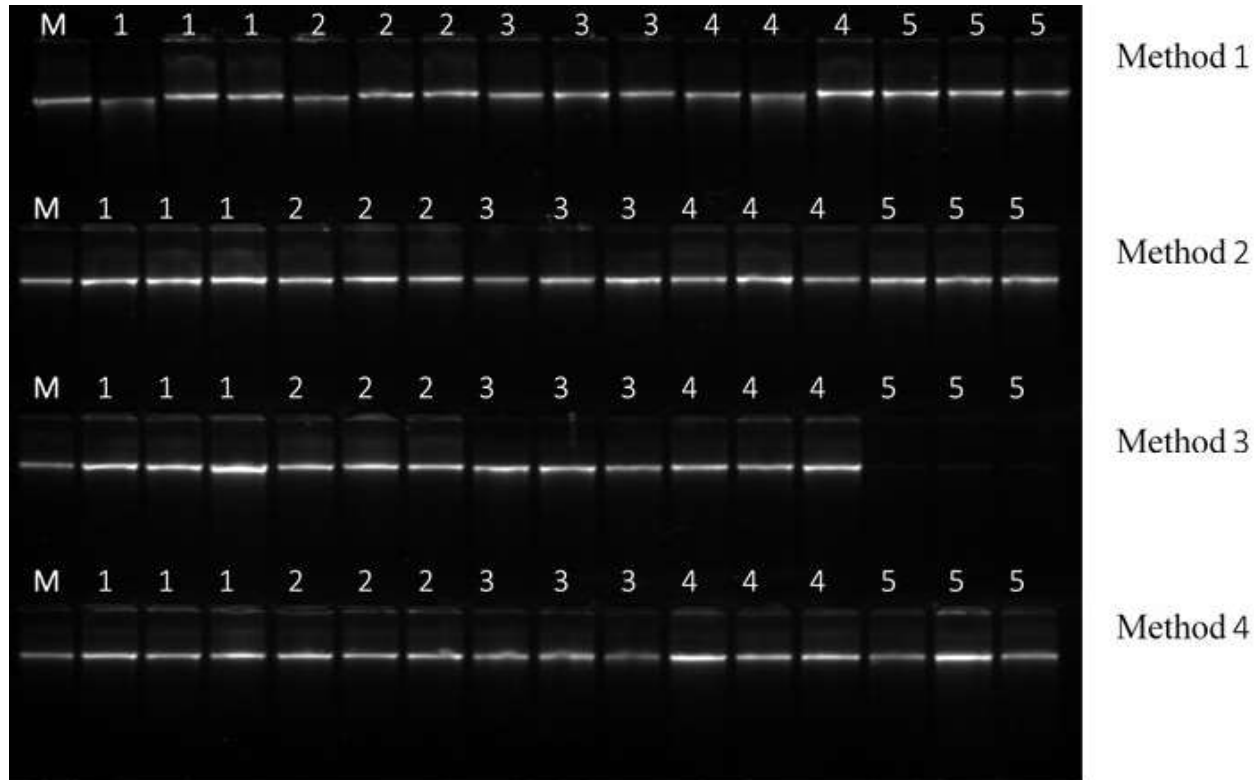


Figure 2. Gel electrophoresis of the total DNA isolated from *H. stricta* (1), *H. wagneriana* (2), *H. bourgaena* (3), *H. collinsiana* (4) and *C. indica* (6) by the different evaluated methods: Method 1 (Haque et al., 2018); Method 2 (Saghai-Marouf et al., 1984); Method 3 (Doyle and Doyle, 1990); Method 4 (Michiels et al., 2003). M = 100 ng of λ DNA uncut (48 and 502 bp). All DNA samples were adjusted to 100 ng of DNA.

DNA molecule. Furthermore, the inclusion of the RNaseA step in all methods, helps to decrease the absorbance of these compounds during the spectrophotometric readings (Tamari et al., 2013; Abdel-Latif and Osman, 2017).

Since the $OD_{A260/A280}$ ratio is only an indication of DNA purity rather than a precise answer, the integrity (taken as DNA of high molecular weight) of the extracted DNA samples was determined by electrophoresis on a 1% agarose gel. Figure 2 shows that all protocols were equally efficient for obtaining high DNA quality and integrity in *Heliconia* spp. (from the qualitative point of view). However, the analysis of variance showed that for *C. indica*, Method 3 was not as efficient as the others. In fact, the DNA yield for this method was the lowest as compared to the other methods (Table 2) and had the highest mean $OD_{A260/A280}$ ratio (2.60 ± 0.11). One possible explanation for this result might be the presence of high levels of phenolic compounds in *C. indica* (Vankar and Srivasta, 2008), and the use of the extraction buffer of Method 3. This buffer did not include PVP and was not as effective as the other buffers for the removal of phenolic compounds. The presence of these compounds in the DNA samples can interfere with the quantification by UV absorption, causing problems during the estimation of the concentrations of the DNA samples and shifting the

$OD_{A260/A280}$ ratio (Mannin, 1991).

DNA functionality

One of the most important features of any DNA extraction protocol is whether the extracted DNA will work in downstream applications. Conventionally, this feature is evaluated digesting the extracted DNA (Barra et al., 2012; Huang et al., 2013; Cavallari et al., 2014). Figure 3 shows the electrophoresis pattern of the DNA samples fully digested with *EcoRI* restriction enzyme. There is an absence of high molecular weight bands, and the continuous characteristic smearing of the different fragment sizes is present. It indicated that the DNA obtained by all the evaluated methods is of high purity and quality. Amplification of the *Hc_D6* locus marker from the total DNA extracted by each method produced a clear and clean target product of 222 to 258 bp in length (Figure 4). This further confirmed the quality of the DNA, free of any inhibitory compounds and suitable for any downstream application (sequencing reactions were also performed successfully, data not shown). At first glance, the DNA extraction protocols described here seem equally efficient in extracting high yield and quality of

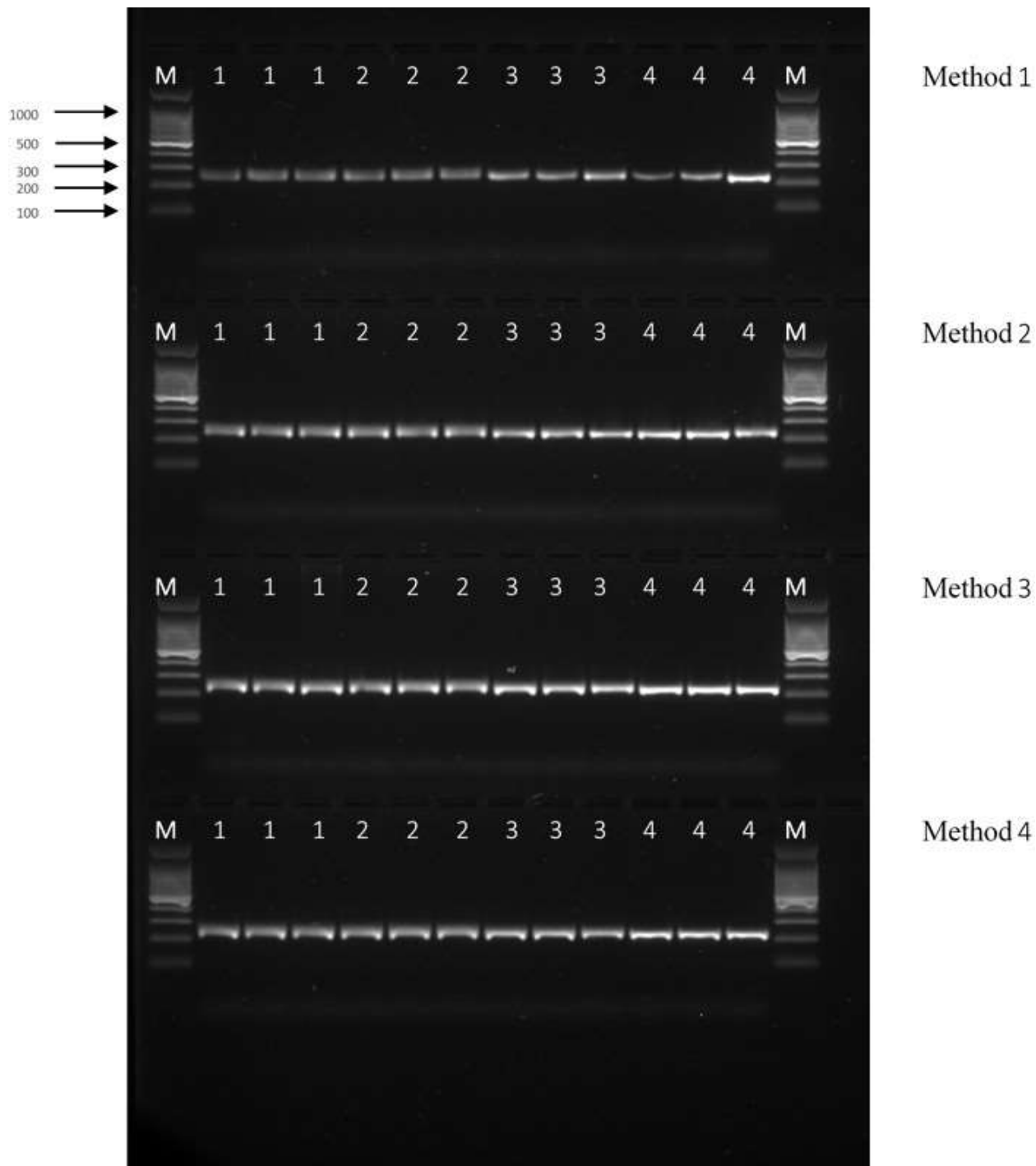


Figure 3. PCR amplification of the *Hc_D6* marker (222-256 bp). M: molecular weight marker (100 bp, DNA ladder, Promega, Madison WI, USA). Only DNA from *Heliconia* spp. was tested for PCR amplification. *H. stricta* (1), *H. wagneriana* (2), *H. bourgaena* (3) and *H. collinsiana* (4) from each evaluated method: Method 1 (Haque et al., 2018), Method 2 (Saghai-Marooof et al., 1984), Method 3 (Doyle and Doyle, 1990) and Method 4 (Michiels et al., 2003).

genomic DNA from the five analyzed species. Yet, once statistical tools were applied into the analysis of results, significant differences were found for both variables. Methods 4 (Michiels et al., 2003) and Method 2 (Saghai-

Marooof et al., 1984) with the modifications assayed in this study, were significantly different for extracting higher DNA yields and better DNA quality. Unlike other protocols, all the steps described here can be carried

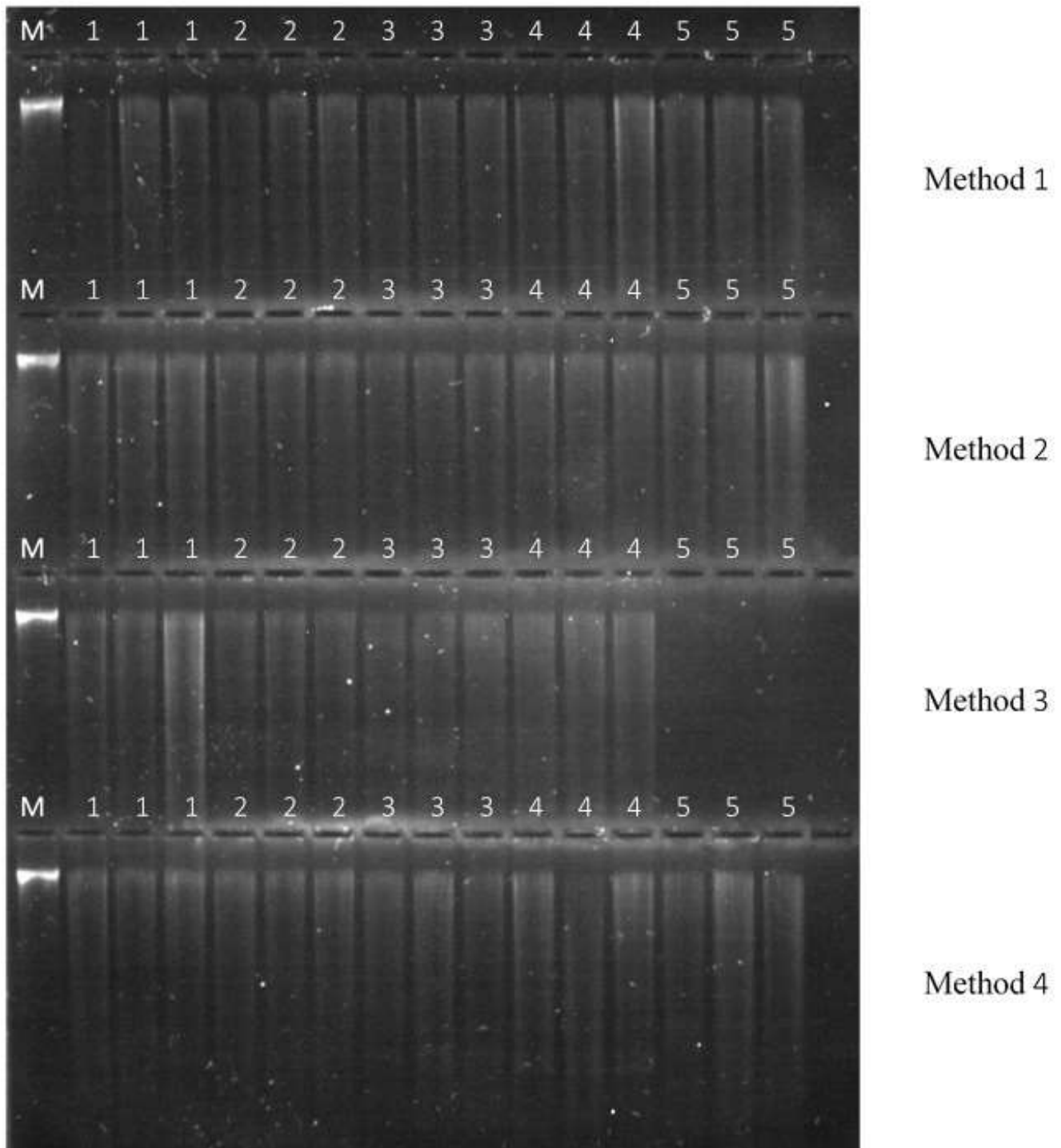


Figure 4. Restriction endonuclease digestion pattern of genomic DNA isolated from *H. stricta* (1), *H. wagneriana* (2), *H. bourgaena* (3), *H. collinsiana* (4) and *C. indica* (6) by the different methods evaluated: Method 1 (Haque et al., 2018), Method 2 (Saghai-Marooft et al., 1984), Method 3 (Doyle and Doyle, 1990) and Method 4 (Michiels et al., 2003). M = 50 ng of undigested genomic DNA.

out at RT, eliminating the need for refrigerated centrifugation or DNA precipitation. Additionally, all the DNA samples were found to be stable when stored at -20°C for more than two years and could be amplified by

PCR.

Although all evaluated protocols were efficient for DNA extraction from mature leaves of *Heliconia* spp., Methods 4 (Michiels et al., 2003) and 2 (Saghai-Marooft et al.,

1984) were statistically superior for obtaining high yield and quality DNA. These methods yielded reproducible amplification of SSR products, demonstrating its compatibility for molecular analysis. These protocols are routinely used in our laboratory to isolate DNA from a wide variety of plant species including *Amaranthus hypocondriacus* L., *Amaranthus cruentus* L., *Agave* species, *Theobroma cacao* L., *Stevia rebaudiana* Bert, *Pinus* species, *Pseudotsuga menziesii* (Mirb) Franco, and *Annona muricata* L., all having high levels of secondary metabolites.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This research was supported by the National Forestry, Crops and Livestock Research Institute of Mexico, through the project "Rescue and Conservation of Heliconias from Southern Mexico".

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