Isolation of high-quality DNA in 16 aromatic and medicinal Colombian species using silica-based extraction columns

Aislamiento de DNA de alta calidad en 16 especies aromáticas y medicinales Colombianas utilizando columnas de extracción de sílica

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ABSTRACT

Aromatic and medicinal plant species are a valuable resource for research and development of pharmaceutical, cosmetic, crop protection and nutritional agents, due to the high amount of bioactive phytochemicals that they contain. However, these compounds are a major obstacle in the isolation of high-quality DNA suitable for genetic analyses. In this paper, we report a protocol that optimizes the use of the cationic detergent CTAB and the reductant β-mercaptoethanol in cell lysis. The elimination of plant secondary metabolites such as polysaccharides and polyphenols, that typically co-isolate with DNA, was achieved using the chemical denaturing properties of the guanidinium cation, which together with the adsorbent chemical specificity of the silica, resulted in the purification of high-quality DNA suitable for digestion with restriction enzymes and optimal for PCR amplification of AFLP-type molecular markers. This protocol was evaluated on 16 Colombian aromatic and medicinal plant species promising for their essential oils. The results allow suggesting that this procedure might be appropriate for other species, tissues and sample types recalcitrant to DNA extraction.

Key words: chaotropic agent, guanidinium cation, silica-based extraction columns, nucleic acid extraction kit, guanidinium salts, molecular markers.

RESUMEN

Las especies vegetales aromáticas y medicinales son un recurso valioso para la investigación y el desarrollo de agentes farmacéuticos, cosméticos, agrícolas y de alimentos, debido a la alta cantidad de fitoquímicos bioactivos que contienen. Sin embargo, estos compuestos son un obstáculo en el aislamiento de ADN de alta calidad adecuado para análisis genéticos. En el presente artículo reportamos un protocolo que optimiza el uso del detergente catiónico CTAB y el agente reductor β-mercaptoetanol en la lisis celular. La eliminación de metabolitos secundarios como polisacáridos y polifenoles que típicamente co-aíslan con el ADN, se logró utilizando las propiedades químicas desnaturalizantes del catión guanidinio que junto con la especificidad química adsorbente de la sílica, resultó en la purificación de ADN de alta calidad adecuado para la digestión por enzimas de restricción y óptimo para la amplificación por PCR de marcadores moleculares tipo AFLPs. Este protocolo se evaluó en 16 especies vegetales aromáticas y medicinales colombianas promisorias por sus aceites esenciales. Los resultados obtenidos permiten sugerir que este procedimiento podría ser apropiado para su aplicación en otras especies, tejidos y tipos de muestras recalcitrantes a la extracción de ADN.

Palabras clave: agente caotrópico, catión guanidinio, columnas de extracción de sílica, kit de extracción de ácidos nucleicos; sales de guanidinio, marcadores moleculares.

Introduction

Worldwide, aromatic and medicinal plant species are used as sources of new molecules for the synthesis of chemopreventive and anti-cancer drugs (Balunas and Kinghorn, 2005), medicinal, cosmetic, antibacterial, antiparasitic (Bakkali *et al*., 2008), antimicrobial, antioxidant and anti-inflammatory agents (Cowan, 1999; Svoboda and Hampson, 1999). Their importance lies in that they contain many bioactive phytochemicals, plant secondary metabolites and essential oils (Balunas and Kinghorn, 2005; Cowan, 1999), significant for the pharmaceutical, crop protection, cosmetic and food industries (Bakkali *et al*., 2008), and certain research areas such as pharmacognosy (Balunas and Kinghorn, 2005), biotechnology, plant breeding, genetics, molecular and evolutionary biology (Canter *et al*., 2005; Gómez-Galera *et al*., 2007; Kumar and Gupta, 2008).

In Colombia, there is an increasing interest in studying the composition and biochemical properties of essential oils from different aromatic and medicinal native plants, and their potential uses (Meneses *et al*., 2009; Nerio *et al*., 2009). The research in this kind of promising species must be accompanied by: *i)* the development of cultivable lines in conventional plant breeding programs and/or assisted

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by molecular markers from wild materials to ensure the proper agronomic exploitation and the conservation of genetic resources (Gómez-Galera *et al.*, 2007; Kumar and Gupta, 2008), *ii)* the application of molecular and genomic tools appropriate for handling the modulation in the production of these oils and plant secondary metabolites (Gómez-Galera *et al.*, 2007), and *iii)* the development of quality control testing to prevent piracy or adulteration of commercial products (Breton *et al.*, 2004; Marieschi *et al.*, 2009).

The use of molecular markers such as AFLPs (Vos *et al.*, 1995), to assist the selection process in plant breeding (Bernardo, 2008; Collard *et al.*, 2005) and quality control procedures (Breton *et al.*, 2004; Marieschi *et al.*, 2009), requires the development of protocols for isolation of high-quality DNA that allows enzymatic digestion with restriction endonucleases and amplification of regions or fragments with high reproducibility (Mace *et al.*, 2003; Matasyoh *et al.*, 2008; Sarwat *et al.*, 2006). However, the isolation of DNA in aromatic and medicinal plant species is a difficult, tedious and time-consuming procedure (Khanuja *et al.*, 1999; Michiels *et al.*, 2003; Padmalatha and Prasad, 2006; Pirttilä *et al.*, 2001). Basically, the problem is the formation of complexes between DNA and plant secondary metabolites such as polyphenols and polysaccharides. Among these metabolites are tannins, flavonoids, quinones, terpenes and alkaloids, that make not only attractive this group of plants but also the procedure of isolation difficult, because they degrade the extracted DNA and directly or indirectly inhibit the action of enzymes used in downstream molecular applications such as PCR amplification (Ivanova *et al.*, 2008; Khanuja *et al.*, 1999; Michiels *et al.*, 2003; Padmalatha and Prasad, 2006; Pirttilä *et al.*, 2001; Porebski *et al.*, 1997).

The commonly reported methods for the extraction of DNA in medicinal and aromatic plants are modifications of classical procedures such as the CTAB of Doyle & Doyle and SDS of Dellaporta (Dellaporta *et al.*, 1983; Doyle and Doyle, 1990), which must be adjusted for each sample type, tissue and species. Variable results are obtained when these procedures are applied in other species, even those closely related (Marva *et al.*, 2007; Pirttilä *et al.*, 2001; Sangwan *et al.*, 2000; Sarwat *et al.*, 2006). Because of this, the aim of this work was to develop a high quality DNA extraction protocol for recalcitrant or difficult samples, that it is chemically simple, fast, reproducible and optimal for aromatic and medicinal plant species, based on the use of chaotropic agents and membrane-silica-based extraction columns.

The use of chaotropic agents for protein denaturation dates back to the work of Svedberg in 1937, and Greenstein in 1938 (Greenstein, 1938; Greenstein, 1939a; Greenstein, 1939b; Greenstein and Edsall, 1940; Svedberg, 1937). Among the chaotropes more commonly used in chemistry and molecular biology for the structural study of proteins and for nucleic acid isolation are the guanidinium cation (Gdm^+) -salts (Bowtell, 1987; Castellino and Barker, 1968; Cox, 1968; Greenstein and Edsall, 1940; Mason *et al.*, 2003). These salts, made up of Gdm^+ or Gdm^+-substituted and an anion such as Cl^-, I^-, Br^- or thiocyanate (SCN^-), constitute the strongest denaturing agents known in nature, and its denaturation strength depends on the accompanying anion, being Cl^- the weakest and the SCN^- the most powerful reported (Castellino and Barker, 1968; Greenstein, 1939b; Greenstein and Edsall, 1940; Mason *et al.*, 2003). Thus, the denaturation or dissociation and solvent properties of these salts on proteins are in function of the type of cation, the accompanying anion and the concentration in solution (Castellino and Barker, 1968; Greenstein, 1939a; Greenstein, 1939b). On the other hand, silica is known for its ability to adsorb specifically nucleic acids (Alexander *et al.*, 2007; Boom *et al.*, 1990; Rogstad, 2003; Sarwat *et al.*, 2006).

Boom *et al.* (1990) reported for the first time the use of Gdm^+ and silica particles jointly in samples of human serum and urine for the isolation of DNA and RNA. This work demonstrated the potentiality of the method, simple chemistry and a basic foundation: in presence of any chaotropic agent and solvent as Gdm^+-salts, nucleic acids are specifically adsorbed by silica particles (Boom *et al.*, 1990; Boom *et al.*, 1999).

The procedure reported in this paper is reproducible, simple, fast, with minimal requirements of specialized equipment and chemistry of easy handling, which proved to be suitable for obtaining high-quality DNA in aromatic and medicinal plant species. This protocol allows recovering on average 4.35 µg of DNA per 150 mg of tissue, with spectral quality of 1.85 for the ratio A_{260}/A_{280} and 2.08 for the ratio A_{260}/A_{230}. The extracted DNA was appropriate for digestion with restriction enzymes, PCR amplification with primers of importance in population genetic studies and amplification of AFLP-type molecular markers.

Materials and methods

Plant material

Young leaves were harvested from 16 medicinal and aromatic Colombian species of importance for its content of essential oils (represented by 20 accessions of interest, see

Tab. 2 below), growing under greenhouse conditions at the Agronomy Faculty at the Universidad Nacional de Colombia, Bogotá. The leaves were placed in an aluminum bag and kept in ice to reduce tissue oxidation until processing.

Buffers and reagents

The protocol of DNA extraction for aromatic species called A-2X is described in detail in Tab. 1. The buffers and reagents used in the procedure are as follows: *i)* Buffer A-2X: 2% CTAB w/v, 1.5 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 1% β-mercaptoethanol (add just before use), *ii)* Chloroform : isoamyl alcohol (24:1, v/v), *iii)* Isopropanol at -20°C, *iv)* Gdm^+-salt solution: *a)* Guanidinium Hydrochloride (GdmHCl) Solution: 2 M or 4 M GdmHCl dissolved in sterile distilled water or *b)* Guanidinium Thiocyanate (GdmSCN) Solution: 2 M or 4 M GdmSCN, *v)* Ethanol at 90 and 70%, *vi)* Low salt TE buffer pH ≥ 8.0, and *vii)* Stock RNase A in TE buffer at a concentration of 1 $\mu g\ \mu L^{-1}$.

Chemistry of the protocol A-2X

The A-2X extraction buffer contains the reagents CTAB, EDTA, NaCl and β-mercaptoethanol. The CTAB is a cationic surfactant used as a detergent for both selective precipitation of nucleic acids and reduction of contamination by polysaccharides and denatured proteins (Cheng *et al.*, 1997; Križman *et al.*, 2006; Michiels *et al.*, 2003; Pirttilä *et al.*, 2001; Sarwat *et al.*, 2006). The EDTA is used as a chelate to inhibit DNases (Matasyoh *et al.*, 2008). The NaCl in high concentrations prevents co-precipitation of DNA together with polysaccharides (Jobes *et al.*, 1995; Križman *et al.*, 2006; Porebski *et al.*, 1997). The β-mercaptoethanol is a reducing agent used to break di-sulfide bridges, inhibit different types of enzymes by destabilization and prevent oxidation of polyphenols (Cheng *et al.*, 1997). The buffer typically has pH between 7.0 and 8.0 for reducing contaminant RNA (Chomczynski and Sacchi, 1987).

The DNA purification is achieved using Gdm^+-salts and silica-based extraction columns. The salts denature and break any inter- and intra-molecular interaction between DNA and proteins. We suggest the use of GdmHCl and GdmSCN, two of the most commonly used salts and with different denaturation powers, the first being the weakest and the other about 2.5 times more powerful (Castellino and Barker, 1968). The silica in the extraction columns efficiently adsorbs high molecular weight DNA after treatment with Gdm^+-salts, which has been used successfully (Alexander *et al.*, 2007; Ding *et al.*, 2008; Ivanova *et al.*, 2008; Sassa, 2007).

Quantification of extracted DNA

Two microliters of each extracted sample were used in order to determine its concentration, quantity and purity in a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc.). Additionally, five microliters of each sample were loaded in a 1% agarose gel to determine the integrity of DNA. The staining of the products were carried out with EZVision™ Three (Amresco®) and each gel was run at 90 V for 30 min. The gels were documented using a gel documentation system Gel Doc XR (Bio-Rad Laboratories Inc.).

Enzymatic digestion with *MseI*

The restriction analysis was performed in nine of the 20 accessions under study. For this, 200 ng of extracted DNA were incubated for each of the samples with 1 U of *MseI* enzyme (Invitrogen™) for 2.5 h, following the manufacturer's instructions. The samples were run at 90 V for 50 min and visualized in a 1% agarose gel.

TABLE 1. Protocol A-2X for DNA isolation from aromatic and medicinal species.

1	Grind 100 to 200 mg of plant tissue with liquid nitrogen in a sterile mortar.
2	Immediately transfer the tissue powder into a sterile 2 mL Eppendorf tube containing 800 µL of buffer A-2X.
3	Homogenize the sample by gently inversion and incubate it for 30 min at 65°C. Shake regularly, *e.g.* every 5 min.
4	Add 800 µL of chloroform: isoamyl alcohol (24:1). Mix the sample by shaking vigorously to achieve an emulsion and centrifuge at 14,400 xg for 10 min.
5	Transfer the supernatant into a sterile 2 mL Eppendorf tube. Add 200 µL of isopropanol at -20°C and mix gently by inversion.
6	Add 1 mL of Gdm^+-salt solution. Quickly, mix by inversion for 5 min.
7	Transfer 700 µL of the sample to a silica-based column (EconoSpin™ All-in-1 Mini Spin Columns, Epoch Biolabs) and centrifuge at 10,000 xg for 5 min. Discard the flow-through by emptying the collection tube and repeat this step until there is no more flow-through.
8	Wash the membrane with 500 µL of 90% ethanol and centrifuge at 10,000 xg for 5 min. Discard the flow-through collected in the collection tube of the column and repeat this step using 70% ethanol.
9	Centrifuge the column at 10,000 xg for 10 min or until the membrane is totally dry. Put the column into a new sterile collection tube.
10	Add 100 µL of low salt TE buffer pH ≥ 8.0 preheated to 65°C directly in the center of the membrane. Incubate at 65°C for 5 min and centrifuge at 10,000 xg for 1 min to collect the flow-through with the DNA.
11	Repeat step 10, but this time incubate only for 3 min.
12	(Optional) Add 2 µL RNase A (1 $\mu g\ \mu L^{-1}$) and incubate at 37°C for 15 min. Check the quality of extracted DNA in a 1% agarose gel.

PCR using primers of importance in genetic population studies

Four pairs of universal primers were used to amplify different non-coding chloroplast regions, *trnL* (UAA) – *trnF* (GAA), *trnL* (UAA) *intron* (Taberlet *et al.*, 1991), *petA* – *psbE* (Fofana *et al.*, 1997), and *trnS* (GCU) – *trnG* (UCC) (Hamilton, 1999), on six of the 20 accessions under study. Each PCR reaction contained 1 U of recombinant *Taq* DNA polymerase (Fermentas Inc.), 1X PCR Buffer with KCl, 4 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer, for a final reaction volume of 25 µL. The reaction was performed on a C1000 Thermal Cycler (Bio-Rad Laboratories Inc.), using the following amplification protocol: 1 cycle at 94°C for 4 min, 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 60 s, and a final extension step at 72°C for 5 min. The products were run at 90 V for 45 min and analyzed by 1% agarose gel electrophoresis.

Amplification of AFLP-type molecular markers

The kit AFLP® Analysis System I (Invitrogen™) was used to amplify three selective primer combinations (E-ACC – M-CAA, E-ACC – M-CAC, and E-ACC – M-CAG), on the same six accessions used previously to amplify non-coding regions of the chloroplast DNA. Two genotypes per accession were taken in order to reveal the presence of polymorphisms. The AFLP amplification was carried out following the manufacturer's instructions. The visualization of the products was performed as recommended by the manufacturer, although the staining of 6% PAGE was performed in silver nitrate. The gel was documented with a digital camera Olympus® SP-350.

Results

The concentration, quantity, quality and usefulness of the DNA extracted with the protocol A-2X were evaluated. The whole extraction process, from the harvesting of tissue until DNA quantification in NanoDrop™ 2000 and visualization in agarose gel on the 16 species (represented by 20 accessions of interest), was repeated three times independently for statistical support (Tab. 2). Similar results in quality and quantity were obtained in all the repetitions performed.

An average of 4.35 µg of DNA with an average concentration of 21.73 ng μL^{-1} was extracted for all species evaluated using 150 mg of fresh tissue, being *L. alba* the species with

TABLE 2. Production and quality data of the DNA extracted using the protocol A-2X in 16 aromatic and medicinal Colombian plant species. The table displays the accession number (CA), species, family, percent concentration of essential oils extracted by hydrodistillation (EO%), average concentration of extracted DNA from 150 mg of initial tissue, standard deviation (SD), and absorbance measurements A_{260} / A_{280} and A_{260} / A_{230}. The columns #PCR and #AFLPs indicate the number of PCR and AFLP reactions that could be carried out using 10 and 100 ng per reaction, respectively. Data shown correspond to the average of three independent repetitions made in the extraction process of the same individual in each accession.

CA	Species	EO (%)	DNA (ng μL^{-1})	SD DNA (ng μL^{-1})	DNA (ng/150 mg)	DNA (μg/150 mg)	A_{260}/A_{280}	A_{260}/A_{230}	# PCR	# AFLPs
				Verbenaceae						
CA-262	*Lippia origanoides*	0.67	23.33	5.67	4,666.11	4.67	1.80	1.87	467	47
CA-261	*Lippia micromera*	0.50	16.87	2.74	3,373.33	3.37	1.82	1.40	337	34
CA-31	*Lippia alba*	0.15	46.46	20.01	9,292.22	9.29	1.90	1.95	929	93
CA-59	*Lantana colombiana*	0.07	25.26	4.26	5,051.11	5.05	1.82	2.15	505	51
				Asteraceae						
CA-10	*Tagetes caracasana*	0.18	16.04	4.56	3,208.89	3.21	1.83	2.35	321	32
CA-45	*Baccharis trinervis*	0.06	24.07	7.01	4,813.33	4.81	1.85	2.23	481	48
CA-19	*Baccharis trinervis*	0.05	29.54	7.74	5,908.89	5.91	1.83	2.43	591	59
CA-02	*Steiractinia helianthoides*	0.03	15.76	9.85	3,151.11	3.15	2.19	2.21	315	32
CA-44	*Chromolaena glomerata*	0.03	14.60	3.96	2,920.00	2.92	1.95	2.29	292	29
CA-30	*Calea tacotana*	0.02	17.88	2.73	3,575.56	3.58	1.93	2.28	358	36
CA-05	*Calea glomerata*	0.01	20.01	4.71	4,002.22	4.00	1.81	1.69	400	40
CA-27	*Baccharis decussata*	0.01	31.97	8.61	6,393.33	6.39	1.84	2.41	639	64
CA-48	*Ayapana turbacensis*	0.01	32.02	5.14	6,404.44	6.40	1.81	2.70	640	64
				Labiatae						
CA-14	*Hyptis sidiifolia*	0.06	15.41	2.34	3,082.22	3.08	1.84	1.85	308	31
CA-26	*Hyptis sidiifolia*	0.05	16.66	3.75	3,331.11	3.33	1.79	2.74	333	33
CA-36	*Marsypianthes chamaedrys*	0.05	23.03	4.73	4,605.56	4.61	1.87	2.04	461	46
CA-39	*Hyptis mutabilis*	0.03	16.61	0.68	3,322.22	3.32	1.81	1.74	332	33
CA-07	*Hyptis pectinata*	0.03	15.76	1.50	3,151.11	3.15	1.75	1.94	315	32
CA-06	*Hyptis mutabilis*	0.02	21.28	5.75	4,255.56	4.26	1.80	1.56	426	43
CA-37	*Hyptis atrorubens*	0.01	12.04	2.80	2,408.89	2.41	1.79	1.77	241	24

the highest amount of DNA (9.29 µg / 150 mg of tissue at a concentration of 46.46 ng μL^{-1}, SD = 20.01 ng μL^{-1}), and *H. atrorubens* the species with the lowest amount (2.41 µg / 150 mg of tissue at a concentration of 12.04 ng μL^{-1}, SD = 2.8 ng μL^{-1}). The average absorbance measurements to qualify the purity of the extracted samples were: A_{260}/A_{280} = 1.85 and A_{260} / A_{230} = 2.08. For each accession the number of PCR and AFLP reactions that could be performed using 10 ng and 100 ng, respectively, were calculated from the total amount of extracted DNA per accession. On average, 434 PCR reactions and 43 AFLP reactions could be carried out from 150 mg of tissue sample using the extraction protocol A-2X.

The quality and the molecular weight of the extracted DNA can be seen in Fig. 1. The DNA is clean in all evaluated species and does not present degradation or residual RNA. Furthermore, if we consider that the lambda DNA sequence is 48.5Kb, the molecular weight of the DNA extracted by the protocol A-2X would be close to the molecular weight of lambda DNA.

On the other hand, the full restriction of DNA with the frequent-cutter enzyme *MseI* resulted in a high production of fragments lower than 2 Kb (Fig. 2). Different non-coding regions of the chloroplast DNA were amplified (Fig. 3), as well as three combinations of selective primers using the kit AFLP® Analysis System I (Invitrogen™) (Fig. 4).

Discussion

The isolation of high quality DNA for genetic analyses in aromatic and medicinal plant species is a difficult and time-consuming procedure (Ivanova *et al.*, 2008; Khanuja *et al.*, 1999; Matasyoh *et al.*, 2008; Michiels *et al.*, 2003; Pirttilä *et al.*, 2001), which is strengthened by the use of conventional protocols that are not easily adaptable to other systems (Marva *et al.*, 2007; Pirttilä *et al.*, 2001; Sangwan *et al.*, 2000; Sarwat *et al.*, 2006). For this reason, we used the potentiality of the chemistry of nucleic acid extraction with Gdm^+-salts and silica (Boom *et al.*, 1990).

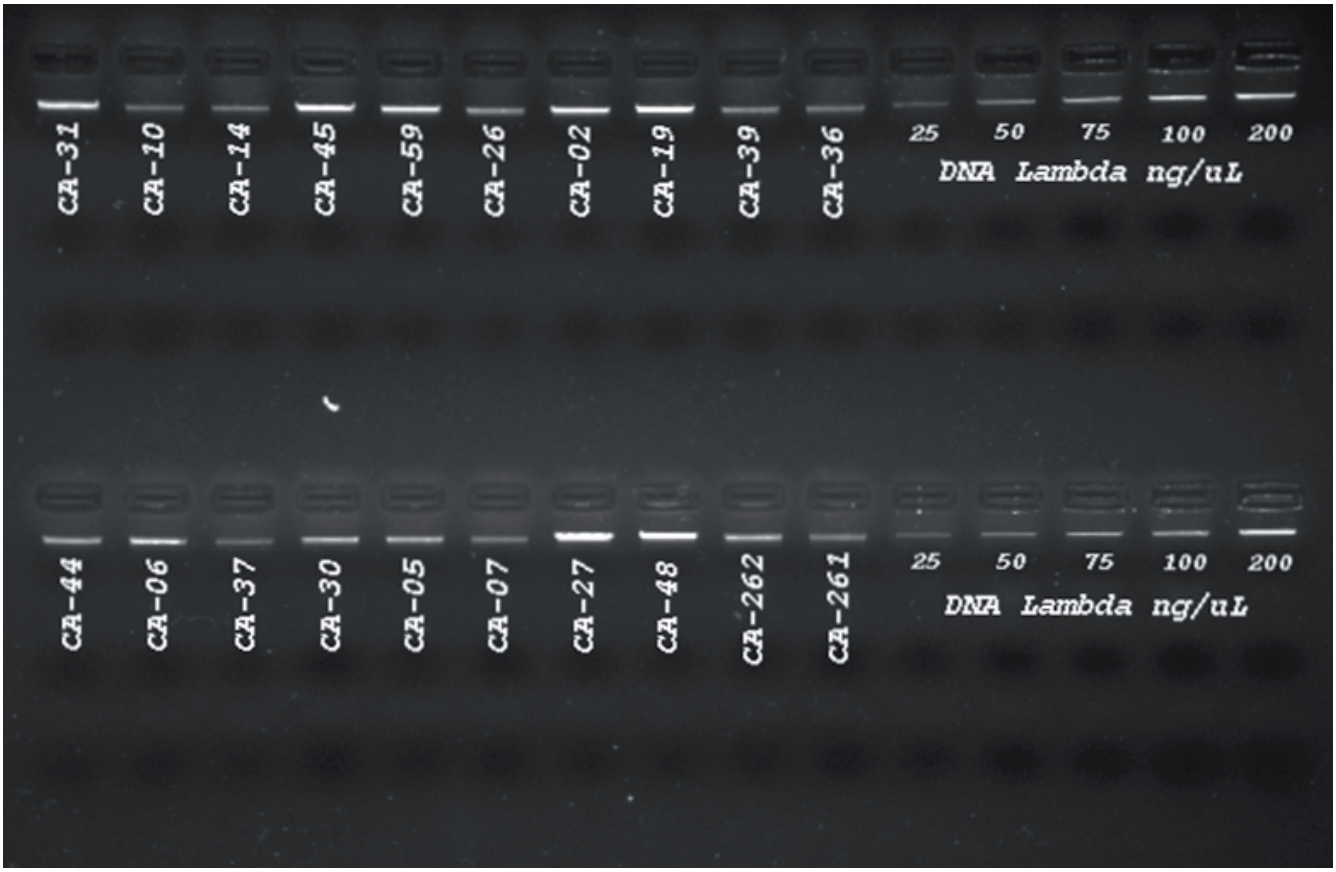

FIGURE 1. Visualization in 1% agarose gel of extracted DNA in 16 medicinal and aromatic Colombian plant species using the protocol A-2X. Right: pattern of lambda DNA (Invitrogen™); the concentrations (ng μL^{-1}) are indicated for comparison. In the samples, CA is the number of accession per species (Tab. 2). Note that the DNA extracted using the protocol A-2X is neither degraded nor contaminated with RNA. The staining was performed with EZVision™.

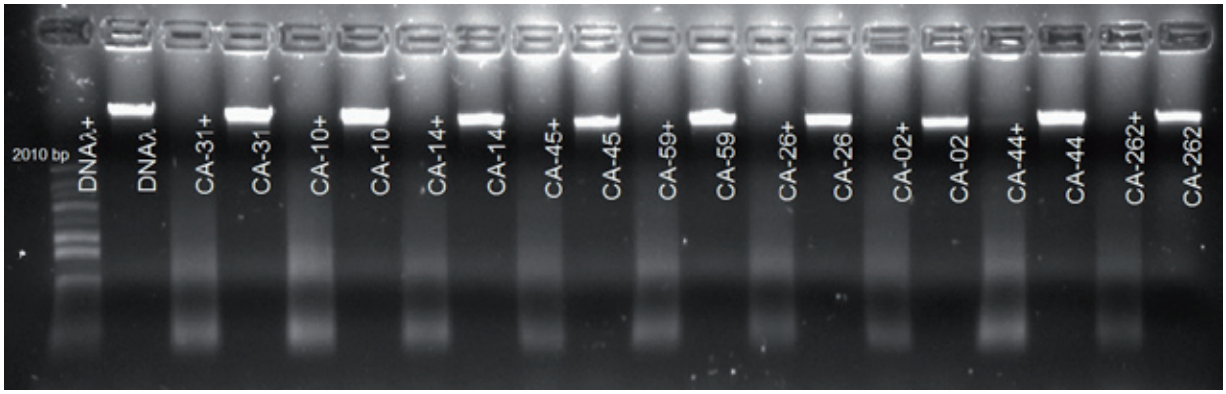

FIGURE 2. Restriction digestion of nine medicinal and aromatic Colombian plant species with the enzyme *MseI*. DNA λ: lambda DNA (Invitrogen™); for the number of accession (CA), see Tab. 2. In each sample, the lane on the left and labeled with (+) corresponds to the restriction with *MseI*, while the other lane corresponds to the negative control. The samples were loaded on a 1% agarose gel and visualized with EZVision™.

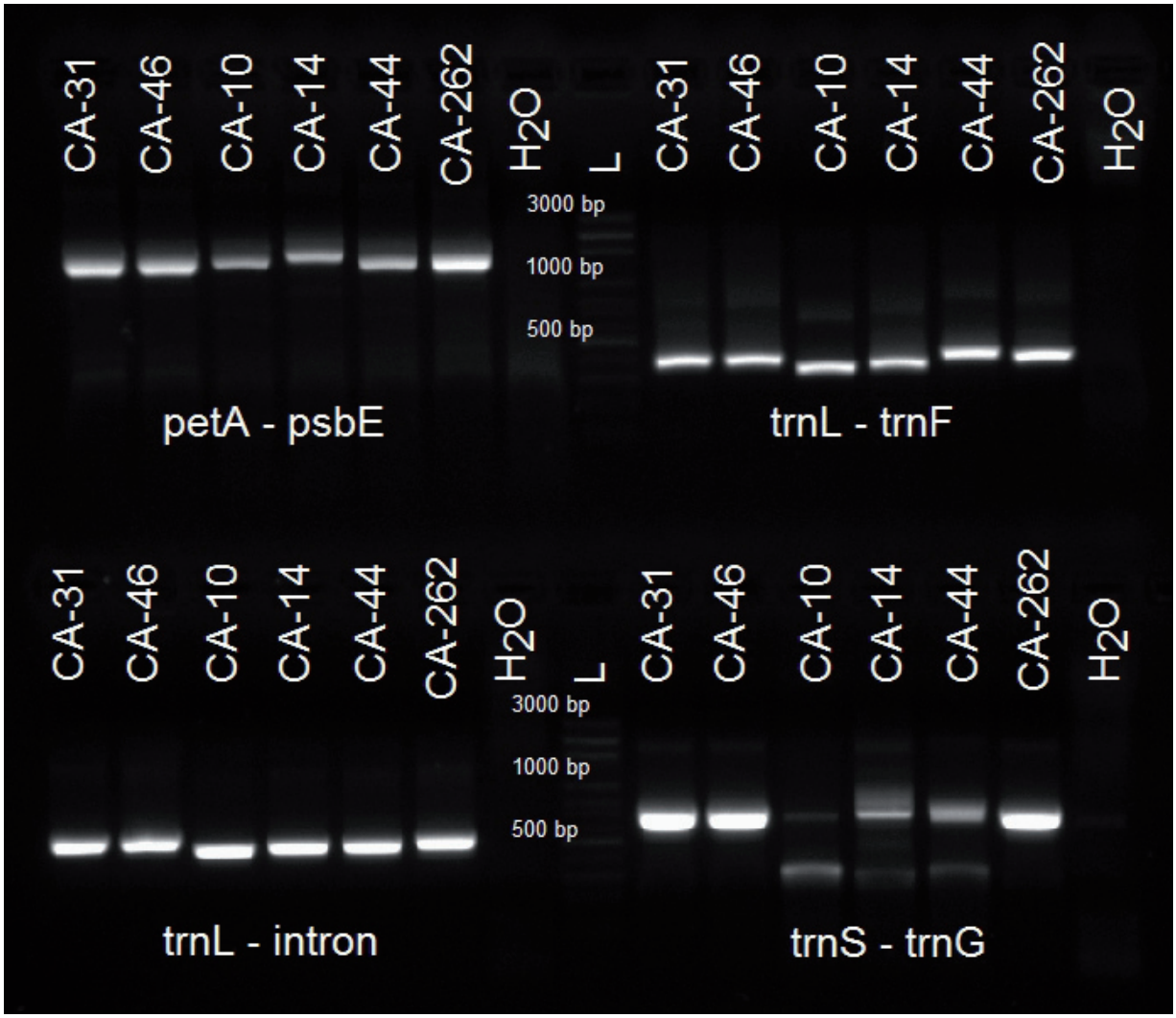

FIGURE 3. PCR amplification of different chloroplast regions in five aromatic and medicinal Colombian species. See materials and methods for references for the primers and regions used. L: molecular weight marker 100 bp Ladder (Axygen Biosciences); for the number of accession (CA), see Tab. 2. The accession CA-46 was included in the amplification because it is a *L. alba* chemotype of interest. Notice that the amplified bands are sharp, and with the exception of the products of CA-10, CA-14 and CA-44 in the region *trnS – trnG*, all the amplicons are suitable for sequencing. The samples were loaded on a 1% agarose gel and visualized with EZVision™.

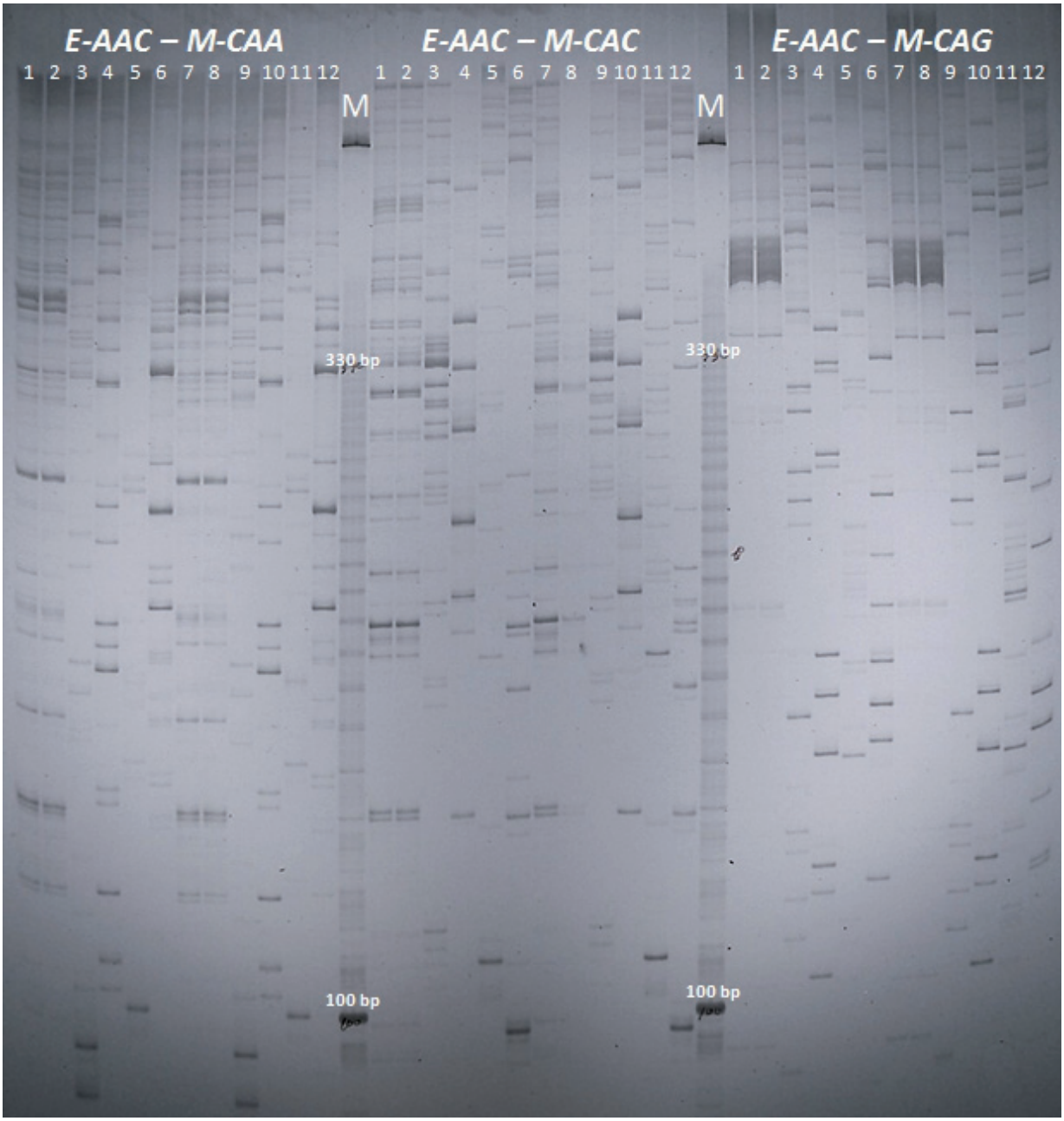

FIGURE 4. AFLP patterns visualized on 6% PAGE for five medicinal and aromatic Colombian plant species using three selective primer combinations from the Kit AFLP® Analysis System I (Invitrogen™). M: molecular weight marker 10bp DNA Ladder (Invitrogen™). Lanes: *L. alba* CA-31, lanes 1 and 7; *L. alba* CA-46, lanes 2 and 8; *T. caracasana* CA-10, lanes 3 and 9; *C. glomerata* CA-44, lanes 4 and 10; *H. sidiifolia* CA-14, lanes 5 and 11; *L. origanoides* CA-262, lanes 6 and 12. Gel staining was performed in silver nitrate.

Using the procedure described in Tab. 1, we were able to extract high quality and high molecular weight DNA in 16 aromatic and medicinal Colombian plant species with variable yields of essential oils (Tab. 2). The DNA extracted by the protocol A-2X is of high molecular weight and high quality which is probably explained by the chemistry used both in the extraction buffer and during the DNA purification (Fig. 1). Furthermore, the use of solutions with Gdm^+ and the nature of the silica-based extraction columns allowed efficient elimination of contaminants and denatured proteins and recovering of large amounts of high molecular weight DNA.

The absorbance measurements of DNA at 280 nm, 260 nm and 230 nm, allowed to calculate two types of ratios widely adopted and qualify the DNA as "pure" or of high-quality (in average, $A_{260}/A_{280} \approx 1.8$ and $A_{260}/A_{230} \approx 2.0$). Nevertheless, some accessions exhibited ratios slightly away from these

values. Digestion with the restriction enzyme *MseI* carried out in nine accessions with variable yields of essential oils and different ratios of purity (Tab. 2), showed that the potential contaminants detected by these ratios did not affect the use of the extracted DNA in restriction digestion and suggest that these ratios *per se* are not reliable or accurate indicators of the usefulness of the extracted DNA in this study. Amplification of four non-coding chloroplast regions in the six evaluated accessions resulted to be successful (Fig. 3) and suitable for sequencing and for subsequent population genetic studies. Likewise, PCR amplification with three selective AFLP primer combinations resulted to be optimal and showed several polymorphisms for each of the accessions evaluated (Fig. 4), which is desirable for the study of genetic variation and the development of molecular markers.

In summary, the DNA extracted using the protocol A-2X met the requirements of quality and quantity to apply different molecular techniques such as PCR, SSRs, RAPDs, ISSRs, AFLPs, sequencing, construction of genomic libraries, among other applications (Mace *et al.*, 2003; Marva *et al.*, 2007; Sarwat *et al.*, 2006). As a more efficient and less expensive alternative to the use of commercial kits for nucleic acid extraction, the protocols that use Gdm^+-salts in conjunction with membranes or particles of silica and report the chemistry of the extraction may be easily implemented in the isolation of DNA from recalcitrant samples (Ivanova *et al.*, 2008).

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