UNIVERSIDAD NACIONAL DE COLOMBIA

Estructura Poblacional de *Lippia origanoides* H.B.K. en el Cañón del Rio Chicamocha (Boyacá & Santander, COL).

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Universidad Nacional de Colombia

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2011

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Trabajo de grado presentado como requisito parcial para optar al título de:

Magister en Ciencias Agrarias

Directora:

Ph.D. María Isabel Chacón Sánchez

Línea de Investigación:

Genética y Fitomejoramiento

Grupo de Investigación:

Horticultura

Universidad Nacional de Colombia

Facultad de Agronomía

Bogotá, Colombia

2011

Este trabajo está dedicado:

A Dios
A mi esposa
A mis padres y hermano

Resumen

Las especies aromáticas y medicinales contienen gran cantidad de fitoquímicos bioactivos útiles para diferentes tipos de industrias, entre estas, agrícola, de alimentos, farmacéutica, cosmética, etc. En Colombia, existe un fuerte interés en estudiar la composición y propiedades bioquímicas de distintas especies aromáticas nativas, además de sus usos potenciales. *Lippia origanoides*, conocida como "orégano de monte", es una especie de la familia Verbenaceae cuyos aceites esenciales han mostrado bioactividad con resultados promisorios contra varios tipos de microorganismos de importancia económica. Sin embargo, a pesar de la potencialidad económica de la especie, poco se conoce sobre sus aspectos básicos biológicos. En este estudio, se utilizaron marcadores moleculares tipo AFLP para determinar la estructura poblacional de la especie en el cañón del rio Chicamocha (COL), y cuantificar los niveles de diversidad genética presentes en las poblaciones inferidas a través de metodologías gráficas de relaciones genéticas y aproximaciones Bayesianas. Se determinó que *L. origanoides* está estructurada en cuatro poblaciones en el cañón: i) LBa y LBb: dos poblaciones pertenecientes a la cuenca baja, ii) MB: población cuenca media, y iii) UB: población cuenca alta. La diferenciación genética entre estas poblaciones fue significativa, con niveles relativamente altos de diversidad; la distribución geográfica de la variación genética de *L. origanoides* se caracterizó por una disminución de la diversidad en la población UB, probablemente debido a condiciones sub-óptimas de desarrollo para la especie en la cuenca alta. La geografía del cañón del rio Chicamocha parece ser relevante en determinar la estructura genética en esta especie.

Palabras clave: Marcadores moleculares tipo AFLP; similitud genética; diferenciación genética; diversidad genética.

Abstract

Aromatic and medicinal species contain high amounts of useful bioactive phytochemicals for different types of industries, among these, agricultural, food, pharmaceutical, cosmetics, etc. In Colombia, there is a strong interest in studying the composition and biochemical properties of various native aromatic species, as well as its potential uses. *Lippia origanoides*, known as "orégano de monte" is a species of the family Verbenaceae whose essential oils have shown promising results with bioactivity against various types of microorganisms of economic importance. In spite of the economic potential of the species, little is known about basic biological aspects. In this study, we used AFLP-type molecular markers to determine the population structure of species in the canyon of the Chicamocha river (COL), and quantify the levels of genetic diversity present in populations inferred by graphical methods of genetic relationships and Bayesian approaches. It was determined that *L. origanoides* is structured in four populations in the canyon: i) LBa and LBb, two populations belonging to the lower basin, ii) MB: middle basin population, and iii) UB: upper basin population. Genetic differentiation among these populations was significant, with relatively high levels of diversity; geographic distribution of genetic variation in *L. origanoides* was characterized by a decrease in diversity in the population UB, probably due to sub-optimal conditions of development for the species in the upper basin. The geography in the canyon of the Chicamocha river seems to be relevant in determining the genetic structure in this species.

Keywords: AFLP-type molecular markers; genetic similarities; genetic differentiation; genetic diversity.

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Introducción general

Análisis detallados del mercado nacional e internacional de los aceites esenciales y aceites vegetales (Biocomercio Sostenible, 2003; Diaz et al., 2002), han revelado que Colombia carece de una participación importante en el mercado, a pesar de contar con amplios recursos vegetales que tienen gran potencial en la producción de este tipo de aceites. La reducida participación en el mercado está determinada por la falta de estrategias, planes, programas, proyectos e incentivos para la producción e investigación en plantas aromáticas, medicinales y condimentarias en nuestro país (Diaz et al., 2002). Así mismo, el bajo o casi nulo conocimiento de la composición genética de las especies aromáticas nativas limita el aprovechamiento de estos recursos como potenciales nuevos cultivos.

Las especies aromáticas y medicinales han sido utilizadas como fuente de nuevas moléculas para la síntesis de drogas quimiopreventivas y anti-cáncer (Balunas y Kinghorn, 2005), de agentes medicinales, cosméticos, bactericidas, anti-parasíticos (Bakkali et al., 2008), antimicrobianos, antioxidantes y antiinflamatorios (Cowan, 1999; Ng et al., 2000; Svoboda y Hampson, 1999), debido a que contienen gran cantidad de fitoquímicos bioactivos, metabolitos secundarios y aceites esenciales (Balunas y Kinghorn, 2005; Cowan, 1999; Gurib-Fakim, 2006), de importancia para la industria farmacéutica, agrícola, cosmética, de alimentos (Bakkali et al., 2008), y diversas áreas de investigación como la farmacognosia (Balunas y Kinghorn, 2005), biotecnología, mejoramiento vegetal, genética y biología molecular (Canter et al., 2005; Gómez-Galera et al., 2007; Kumar and Gupta, 2008), explotación agronómica y conservación de los recursos genéticos (Gómez-Galera et al., 2007).

En Colombia, hay un creciente interés en estudiar la composición y propiedades bioquímicas de los aceites esenciales de diferentes plantas aromáticas y medicinales nativas, y sus potenciales usos (Meneses et al., 2009a; Nerio et al., 2009). Se destaca la especie conocida como “Orégano de monte”, *Lippia origanoides* H. B. K. Esta especie perteneciente a la familia Verbenaceae, es un arbusto aromático, erecto y ramificado que alcanza hasta 3m de altura, de hojas simples y opuestas con tamaños variables debido a

posibles adaptaciones fisiológicas y morfológicas en respuesta a la exposición a la luz (Parra y Rodríguez, 2007), con numerosas inflorescencias caracterizadas por pequeñas flores blancas pediceladas de 4mm de tamaño (de Campos et al., 2011), y una alta producción de frutos secos y semillas por planta. Los aceites esenciales extraídos de esta planta, han presentado actividades antioxidantes, antimicrobianas, antigenotóxicas, antiparasíticas (Arcila-Lozano et al., 2004; Pascual et al., 2001), debido a que *L. origanoides* cuenta con una importante variación fitoquímica representada en tres quimiotipos según el compuesto mayoritario presente en sus aceites esenciales (A: *p-cimeno*, B: *carvacrol*, C: *timol*) (dos Santos et al., 2004; Oliveira et al., 2007; Stashenko et al., 2008; Stashenko et al., 2010). La utilidad de los extractos y aceites esenciales de estos quimiotipos ha sido evaluada con resultados promisorios en microorganismos de importancia agrícola y de salud humana (dos Santos et al., 2004; Henao et al., 2009; Oliveira et al., 2007; Ramírez et al., 2009), actividad antioxidante (Celis et al., 2007; Muñoz-Acevedo et al., 2009), repelente contra *Sitophilus zeamais* y *Tribolium castaneum* (Nerio et al., 2009; Olivero-Verbel et al., 2009), efecto antiviral (Meneses et al., 2009a; Meneses et al., 2009b), y efecto protector sobre el ADN contra genotoxicidad inducida por bleomicina (Vicuña et al., 2010). Estos resultados permiten señalar que los extractos y aceites esenciales de *L. origanoides* son de amplio espectro (Celis et al., 2007; Stashenko et al., 2010), apropiados como fuente de fitomedicinas y con usos potenciales en diversas industrias como alimentos, cosmética, farmacológica, agrícola, etc. (Bueno-Sánchez et al., 2009; Stashenko et al., 2010). Pese a la potencialidad de la especie, es escasa la investigación básica en *L. origanoides*, razón por lo cual es necesario realizar diferentes tipos de estudios, por ejemplo, biología reproductiva, ploidía, niveles de diversidad genética inter e intra-poblacional, etc. Esta información permitirá el desarrollo de un programa de mejoramiento para la adecuada explotación de la especie y su conservación (Stashenko et al., 2010).

L. origanoides, al igual que muchas especies aromáticas, constituye un reto en laboratorio puesto que presenta una alta cantidad de compuestos y metabolitos secundarios que potencialmente dificultan o limitan la aplicación de diferentes técnicas moleculares, entre éstas la extracción de ADN de alta calidad, de ARN no degradado, PCR (Polymerase Chain Reaction), digestión por enzimas de restricción, amplificación de regiones genómicas de interés o de marcadores moleculares tipo RAPDs (Random Amplified Polymorphic DNA), AFLPs, microsatélites, análisis Southern Blot, etc. (Marva et al., 2007; Moyo et al., 2008; Pirttilä et al., 2001),

En este estudio, el objetivo general de la investigación fue determinar la estructura poblacional de *L. origanoides* en el Cañón del rio Chicamocha, utilizando marcadores moleculares tipo AFLP. La implementación de los AFLP demandó desarrollar un protocolo para la extracción de ADN de alta calidad para especies aromáticas consideradas como recalcitrantes por la presencia de altas cantidades de metabolitos secundarios que co-precipitan con el ADN o inhiben reacciones enzimáticas posteriores (esta investigación está en proceso de publicación, Artículo I). Un total de 64 combinaciones de primers selectivos AFLP fueron evaluadas para determinar un grupo de cinco combinaciones útiles en la cuantificación de la diversidad genética e inferencia de la estructura poblacional en *L. origanoides* (los resultados de la evaluación están en proceso de publicación, Articulo II). Entre Agosto y Noviembre de 2009, se realizó un muestreo amplio de la especie en el cañón del rio Chicamocha (cuenca alta, media y baja), entre los departamentos de Santander y Boyacá, Colombia. En total, 173 individuos fueron recolectados para realizar un análisis de diversidad y estructura genética utilizando cinco combinaciones AFLP previamente seleccionadas (Los resultados de la investigación están en proceso de publicación, Articulo III). El análisis desarrollado indicó que *L. origanoides* exhibe niveles relativamente altos de diversidad genética en el cañón del rio Chicamocha, además, la especie se encuentra estructurada en cuatro grupos genéticamente homogéneos o poblaciones, las cuales están distribuidas discretamente a través de la geografía y el gradiente altitudinal presente en las diferentes cuencas del rio.

Los avances realizados en este trabajo (1- Desarrollo de un protocolo de extracción de ADN de alta calidad óptimo para especies aromáticas, 2- Establecimiento, evaluación y selección de combinaciones selectivas de cebadores AFLP para el estudio genético de *L. origanoides*, y 3- Determinación de la estructura genética y cuantificación de la diversidad en la región del cañón del rio Chicamocha), constituyen el primer avance en el conocimiento sobre el estado actual de la diversidad genética y la estructura poblacional de *L. origanoides* en la región del Cañón del rio Chicamocha, la cual alberga además, la mayor cantidad de la variación fitoquímica presente en la especie. Se espera que a mediano plazo, estos resultados contribuyan fuertemente al desarrollo de un programa de mejoramiento genético de especies aromáticas que permita la explotación adecuada de los recursos fitogenéticos presentes en el cañón del rio Chicamocha, además de su conservación y protección, fortaleciendo la cadena productiva de especies aromáticas, medicinales, condimentarias y afines, y la línea de investigación de “Recursos Genéticos

y Fitomejoramiento en Especies Hortícolas" del grupo de Horticultura de la Universidad Nacional de Colombia, UNAL (Bogotá).

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1. Isolation of High-Quality DNA in 16 Aromatic and Medicinal Colombian Species Using Silica-Based Extraction Columns.

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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 plant species promising for their essential oils. The results allow us to suggest that this procedure might be appropriate for other species, tissues and sample types recalcitrant to DNA extraction.

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 colombianas promisorias por sus aceites esenciales. Los resultados obtenidos nos 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.

Keywords: chaotropic agent; guanidinium cation; silica-based extraction column; nucleic acid extraction kit; guanidinium salts; molecular markers.

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, anti-parasitic (Bakkali et al., 2008), antimicrobial, antioxidant and antiinflammatory 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 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 us to recover 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 Table 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 Table 1. The buffers and reagents used in the procedure are as follow. 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 µg/µL.

Chemistry of the protocol A-2X

The extraction buffer designated A-2X 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 polysaccharide (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 hours, following the manufacturer's instructions. The samples were run at 90 V for 50 min and visualized 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 MgCl2, 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, 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 (Olympus®).

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 (Table 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 was extracted for all species evaluated using 150 mg of fresh tissue, being *L. alba* the species whit the highest amount of DNA (9.29 µg / 150 mg of tissue at a concentration of 46.46 ng/µL, SD = 20.01 ng/µL), and *H. atrorubens* the species with the lowest amount (2.41 µg / 150 mg of tissue at a concentration of 12.04 ng/µL, SD = 2.8 ng/µL). 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 (see Fig. 2). Different non-coding regions of the chloroplast DNA were amplified (see Fig. 3), as well as three combinations of selective primers using the kit AFLP® Analysis System I (Invitrogen™) (see 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).

Using the procedure described in Table 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 (see Table 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 (see 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 us 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 $A_{260}/A_{280} \approx 1.8$ or $A_{260}/A_{230} \approx 2.0$. Digestion with the restriction enzyme *MseI* carried out in nine accessions with variable yields of essential oils and different ratios of purity (see Table 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).

Acknowledgments

This work was funded by the Ministerio de Agricultura y Desarrollo Rural (MADR) of Colombia, contract No. 064-2007V7163-50-07, the Universidad Nacional de Colombia and COLCIENCIAS through a research fellowship under the “Programa de Jóvenes Investigadores e Innovadores" contract No. 155-2008.

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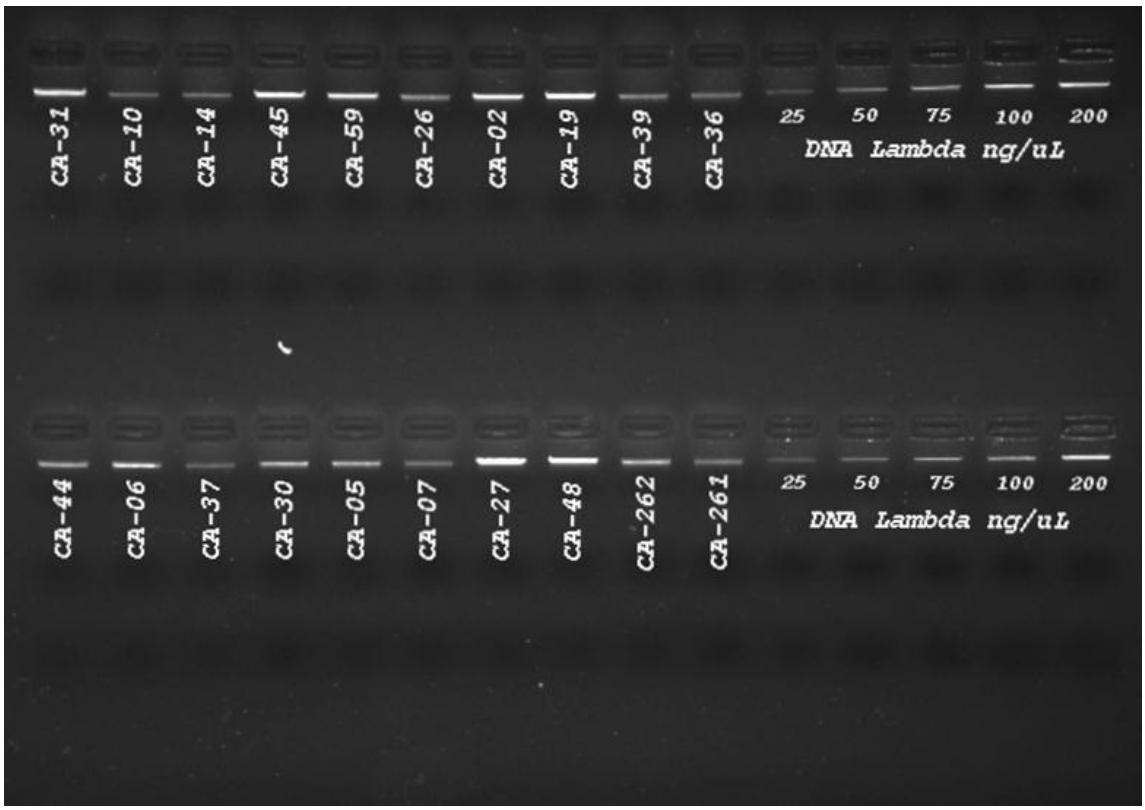

Fig. 1. Visualization in 1% agarose gel of extracted DNA in 16 medicinal and aromatic Colombian plant species using the protocol A-2X. Right: pattern of lambda DNA (Invitrogen™), the concentrations (ng/μL) are indicated for comparison. In the samples, CA is the number of accession per species (see Table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

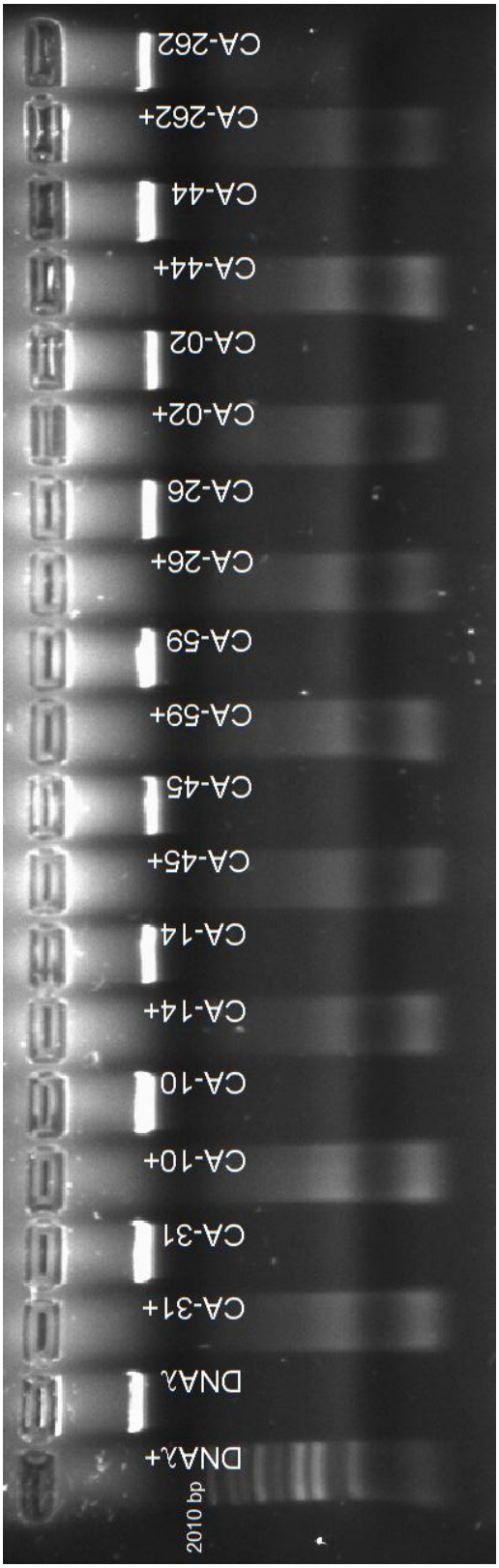

Fig. 2. Restriction digestion of nine medicinal and aromatic Colombian plant species with the enzyme *MseI*. DNAλ: lambda DNA (Invitrogen™); for the number of accession (CA), see Table 2. In each sample, the lane on the left and labeled with (+) corresponds to the restriction with *MseI*, while the other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

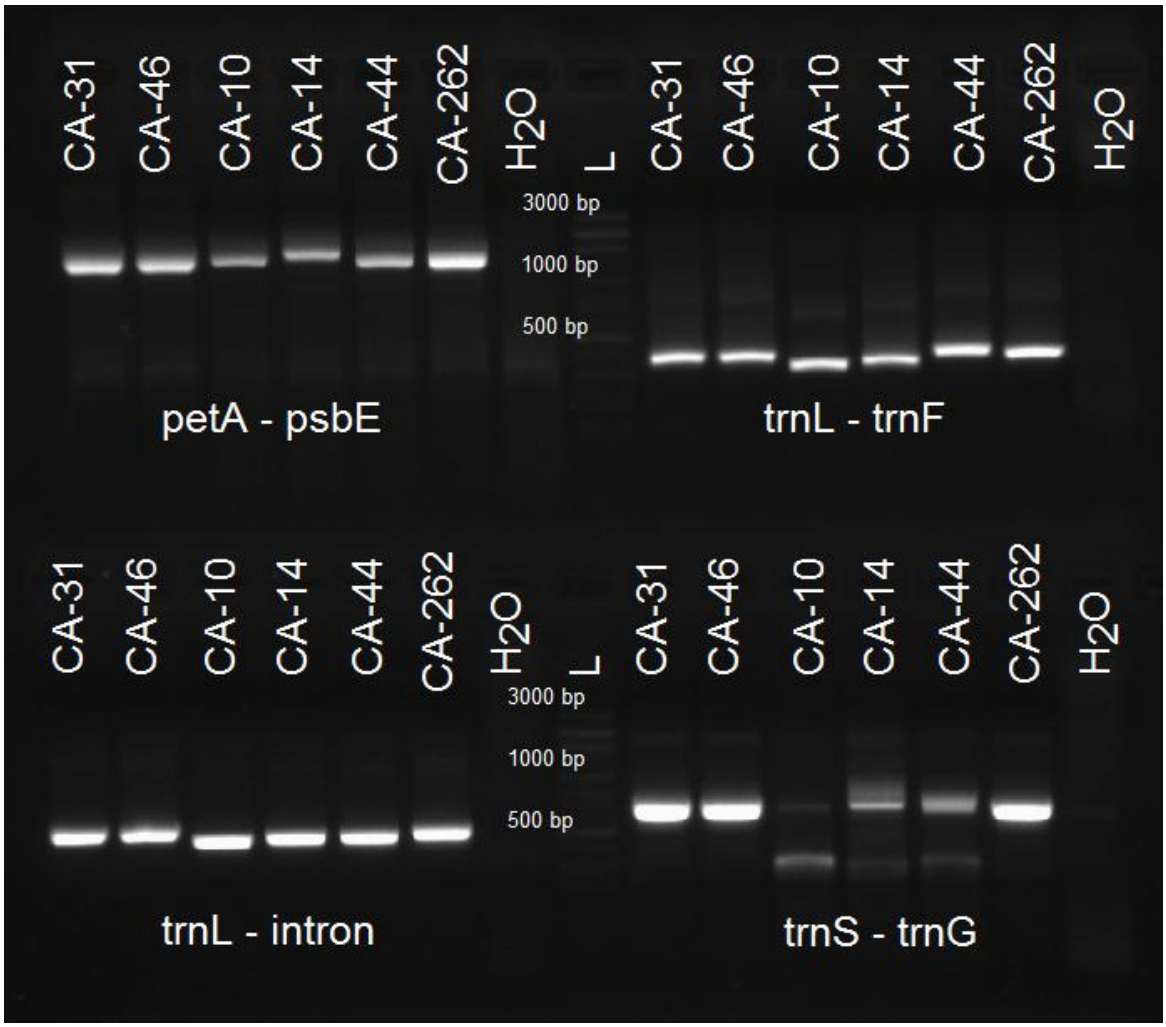

Fig. 3. PCR amplification of different chloroplast regions in five aromatic and medicinal Colombian species. See materials and methods for description of the primers and regions used. L: molecular weight marker 100bp Ladder (Axygen Biosciences); for the number of accession (CA) see Table 2. The accession CA-46 was included in the amplification because it is a *L. alba* chemotype of interest. Notice that the amplified bands are defined, 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 ™.

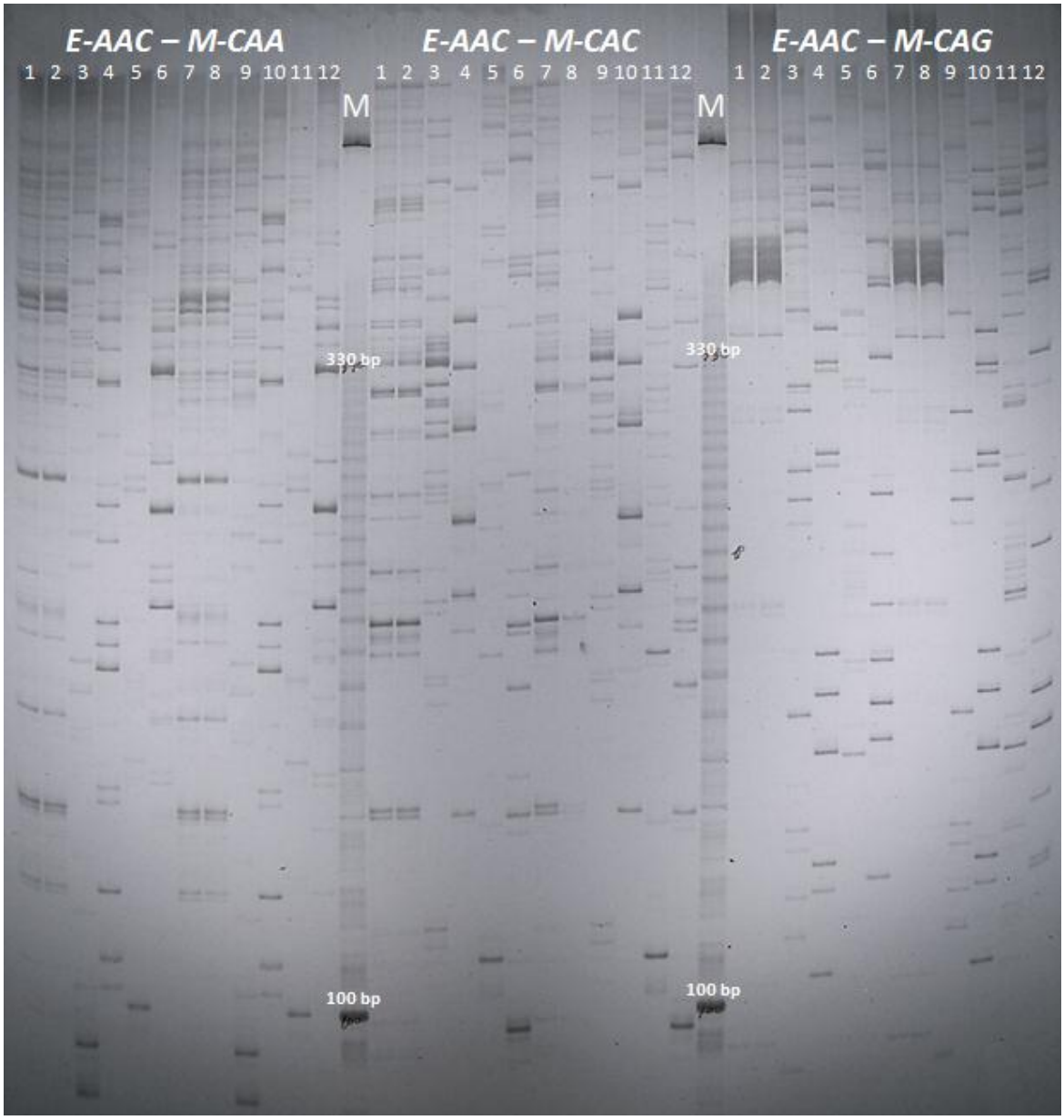

Fig. 4. AFLP patterns visualized on 6% PAGE for five medicinal and aromatic Colombian species using three selective primer combinations from the Kit AFLP® Analysis System I (Invitrogen™). M: molecular weight marker 10bp DNA Ladder (Invitrogen™). Lanes: *L. alba* CA-31, lanes 1 and 7; *L. alba* CA-46, 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.

Table 1. Protocol A-2X.

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 14400 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 10000 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 10000 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 10000 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 10000 xg for 1 min to collect the flow-through with the DNA
11. Repeat step 10, but this time incubate for only 3 min.
12. (Optional) Add 2 µL RNase A (1 µg/µL) and incubate at 37°C for 15 min. Check the quality of extracted DNA in a 1% agarose gel

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 150mg of initial tissue, standard deviation (SD), and absorbance measurements A260/A280 and A260/A230. The columns #PCR and #AFLPs indicate the number of PCR and AFLP reactions that could be carried out using 10ng and 100ng 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	SD [DNA] ng/µL	[DNA] ng/150mg	[DNA] µg/150mg	A_{260}/A_{280}	A_{260}/A_{230}	# PCR	# AFLPs
Verbenaceae										
CA-262	*L. origanoides*	0.67	23.33	5.67	4666.11	4.67	1.80	1.87	467	47
CA-261	*L. micromera*	0.50	16.87	2.74	3373.33	3.37	1.82	1.40	337	34
CA-31	*L. alba*	0.15	46.46	20.01	9292.22	9.29	1.90	1.95	929	93
CA-59	*L. colombiana*	0.07	25.26	4.26	5051.11	5.05	1.82	2.15	505	51
Asteraceae										
CA-10	*T. caracasana*	0.18	16.04	4.56	3208.89	3.21	1.83	2.35	321	32
CA-45	*B. trinervis*	0.06	24.07	7.01	4813.33	4.81	1.85	2.23	481	48
CA-19	*B. trinervis*	0.05	29.54	7.74	5908.89	5.91	1.83	2.43	591	59
CA-02	*S. helianthoides*	0.03	15.76	9.85	3151.11	3.15	2.19	2.21	315	32
CA-44	*C. glomerata*	0.03	14.60	3.96	2920.00	2.92	1.95	2.29	292	29
CA-30	*C. tacotana*	0.02	17.88	2.73	3575.56	3.58	1.93	2.28	358	36
CA-05	*C. glomerata*	0.01	20.01	4.71	4002.22	4.00	1.81	1.69	400	40
CA-27	*B. decussata*	0.01	31.97	8.61	6393.33	6.39	1.84	2.41	639	64
CA-48	*A. turbacensis*	0.01	32.02	5.14	6404.44	6.40	1.81	2.70	640	64
Labiatae										
CA-14	*H. sidiifolia*	0.06	15.41	2.34	3082.22	3.08	1.84	1.85	308	31
CA-26	*H. sidiifolia*	0.05	16.66	3.75	3331.11	3.33	1.79	2.74	333	33
CA-36	*M. chamaedrys*	0.05	23.03	4.73	4605.56	4.61	1.87	2.04	461	46
CA-39	*H. mutabilis*	0.03	16.61	0.68	3322.22	3.32	1.81	1.74	332	33
CA-07	*H. pectinata*	0.03	15.76	1.50	3151.11	3.15	1.75	1.94	315	32
CA-06	*H. mutabilis*	0.02	21.28	5.75	4255.56	4.26	1.80	1.56	426	43
CA-37	*H. atrorubens*	0.01	12.04	2.80	2408.89	2.41	1.79	1.77	241	24

2. Evaluación de Marcadores Moleculares tipo AFLPs en Tres Especies Aromáticas y Medicinales Colombianas con Altos Contenidos de Aceites Esenciales.

Nelson Enrique Vega-Vela, María Isabel Chacón Sánchez.

Resumen

El desarrollo de estrategias para la selección de combinaciones selectivas de cebadores AFLP ha sido un área poco explorada para este sistema de marcadores moleculares, a pesar de la gran importancia que representa la selección óptima de las combinaciones AFLP para obtener un muestreo amplio del genoma. En especies no-modelo, esta información es casi inexistente y pocos artículos reportan estrategias, criterios de selección o los resultados obtenidos para cada una de las combinaciones AFLP evaluadas. En este estudio, se evaluaron 64 combinaciones de cebadores AFLPs utilizando una estrategia de *screening* rápida para seleccionar las combinaciones más apropiadas para estudios genéticos en tres especies aromáticas y promisorias por la calidad de sus aceites esenciales: i) *Lippia origanoides*, ii) *Lippia alba* y iii) *Tagetes caracasana*. Amplificación de cada una de las combinaciones AFLP utilizando dos individuos genéticamente heterogéneos por especie, permitió obtener información suficiente para seleccionar cinco combinaciones útiles en estudios de diversidad y diferenciación poblacional para cada una de las especies. Los resultados de la evaluación inicial para todas las combinaciones AFLP utilizadas, son reportados explícitamente para permitir que otros investigadores puedan seleccionar un número mayor de combinaciones según el alcance y la resolución necesaria para abordar diversas preguntas de investigación.

Abstract

The development of strategies for the selection of AFLP combinations of selective primers has been an unexplored area, in spite of the great importance that represents the optimal selection of AFLP combinations for genome-wide sampling, in a genetic study. In non-model species, this information is almost absent and few articles report strategies, selection criteria or results obtained for each one of the AFLP combinations evaluated. In this study, we assessed 64 combinations of AFLP primers using a rapid screening strategy to choose the most appropriate combinations for genetic studies in three aromatics and promising species for the quality of its essential oils: i) *Lippia origanoides*, ii) *Lippia alba* and iii) *Tagetes caracasana*. Amplification of each AFLP combinations using two genetically heterogeneous individuals per species allowed us to obtain sufficient information to select five combinations useful in studies of diversity and population differentiation for each species. The results of the initial assessment for all used AFLP combinations are reported explicitly to enable than others researchers choose a different and larger number of AFLP combinations depending on the scope of resolution needed to address research questions.

Palabras clave: DNA fingerprinting; enzimas de restricción; motivos de restricción; relaciones genéticas; diversidad genética.

Introducción

La técnica de fingerprinting conocida como AFLP, patentada en 1991 por KeyGene N. V. (Zabeau and Vos, 1993) y descrita por Vos et al (1995), es un sistema de marcadores moleculares con herencia de tipo dominante que permite evaluar la presencia o ausencia de fragmentos genómicos de restricción determinados principalmente por la distribución específica de los motivos de restricción en el genoma (Blears et al., 1998; Meudt and Clarke, 2007; Savelkoul et al., 1999; Vos et al., 1995). La palabra AFLP acuñada a este sistema ha sido mal interpretada debido a su uso erróneo como acrónimo (generalmente en inglés AFLP corresponde con *Amplified Fragment Length Polymorphism*), generando un error conceptual sobre el tipo de polimorfismo que esta técnica logra detectar. Vos et al (1995) en su publicación señalaron claramente que los AFLPs no pueden detectar diferencias en la longitud de los fragmentos provenientes de un mismo locus, por

ejemplo, dos fragmentos provenientes de loci distintos pueden presentar el mismo tamaño molecular debido a una inserción o a una supresión (conocido como indel, abreviatura del inglés insertion/deletion), razón por la cual este sistema presenta importantes restricciones como homoplasia por tamaño de fragmento (Caballero y Quesada, 2010; Caballero et al., 2008; Vekemans et al., 2002). De lo anterior, es posible evaluar únicamente la presencia o la ausencia de una banda o fragmento de restricción amplificado selectivamente para un peso molecular específico y asumiendo que estas bandas, a través de los individuos bajo estudio, provienen del mismo locus en el genoma. A pesar de esta importante restricción y de otras como su herencia de tipo dominante, incapacidad para identificar marcadores homólogos, dificultad en su automatización, bajo nivel de información por locus, etc. (Meudt y Clarke, 2007; Mueller y Wolfenbarger, 1999; Schlötterer, 2004), los AFLPs presentan enormes ventajas como implementación rápida, económica, confiable y fácil sobre cualquier tipo de organismo sin información previa de su genoma, alta reproducibilidad y sensibilidad para detectar regiones altamente polimórficas, muestreo amplio y aleatorio del genoma, alto contenido de información, entre otras (Blears et al., 1998; Meudt y Clarke, 2007; Savelkoul et al., 1999); estas ventajas resultan ser suficientes para hacer de éste un sistema de marcadores moleculares atractivo para su establecimiento en especies no-modelo (Mariette et al., 2002; Meudt y Clarke, 2007).

Metodológicamente, esta técnica y sus modificaciones para la detección de diferentes tipos de polimorfismos han sido revisadas ampliamente (Bensch y Åkesson, 2005; Blears et al., 1998; Meudt y Clarke, 2007; Savelkoul et al., 1999), al igual que su gran utilidad en diversas áreas de investigación. La visualización de los AFLPs en geles de poliacrilamida por tinción con plata, ha sido implementada con éxito en diferentes estudios (Chalhoub et al., 1997; Karam et al., 2006), ya que permite observar un perfil o patrón único de bandas correspondiente al producto de la amplificación selectiva de un conjunto específico de fragmentos de restricción, recuperar y re-amplificar fragmentos específicos, entre otras (Chalhoub et al., 1997; McLenachan et al., 2000).

El *screening* o tamizaje para la selección precisa de las combinaciones selectivas de cebadores y polimórficas en este sistema de marcadores, es una labor importante debido a las limitaciones y desventajas que presentan los AFLPs. En diferentes tipos de estudios genéticos se realizan grandes esfuerzos para tener representada adecuadamente la variación entre poblaciones y entre individuos dentro de poblaciones; no obstante, poco se invierte en el muestreo de la variación entre loci dentro de individuos (Mariette et al.,

2002), lo cual está determinado en el caso de los AFLPs, por la selección precisa de combinaciones selectivas de cebadores que muestreen aleatoriamente todo el genoma. Se han sugerido algunos criterios de selección para combinaciones de cebadores AFLP (Meudt y Clarke, 2007), por ejemplo, i) número total de bandas, ii) porcentaje de polimorfismo por combinación de cebadores, y iii) reproducibilidad del perfil AFLP. Sin embargo, en la literatura existen pocas publicaciones que reporten estrategias, criterios de selección y resultados del tamizaje de combinaciones selectivas de cebadores AFLP en diferentes especies, limitando la implementación y aprovechamiento de todo el potencial que ofrece esta técnica. No obstante, algunas consideraciones pueden mejorar la aplicación de la técnica: i) asegurar una calidad adecuada del ADN extraído, ii) visualización y calificación adecuada del patrón observado, iii) selección de un rango apropiado de lectura por tamaño del fragmento, y iv) selección de diferentes combinaciones de cebadores con un nivel moderado de marcadores o bandas (Benjak et al., 2006; Caballero et al., 2008; Chalhoub et al., 1997; Vekemans et al., 2002).

El objetivo de este trabajo fue evaluar un conjunto de combinaciones de cebadores AFLPs utilizando una metodología de tamizaje rápido, permitiendo seleccionar las combinaciones de cebadores más apropiadas para estudios poblacionales y de diversidad genética bajo diferentes criterios de selección tales como: i) número de bandas totales, ii) número de bandas indiscutibles, iii) porcentaje de polimorfismo y iv) reproducibilidad del perfil AFLP. Para el desarrollo de este estudio se utilizaron tres especies aromáticas colombianas promisorias por la calidad de sus aceites esenciales: *Lippia origanoides*, *Lippia alba* y *Tagetes caracasana*.

Materiales y métodos

Material Vegetal

Se cosecharon hojas jóvenes de diferentes accesiones de tres especies aromáticas promisorias por la calidad de sus aceites esenciales: i) *L. origanoides*, ii) *L. alba* y iii) *T. caracasana*. Los materiales evaluados se encuentran cultivados bajo condiciones de invernadero en la Facultad de Agronomía de la Universidad Nacional de Colombia. Las hojas cosechadas fueron cortadas, colocadas inmediatamente en bolsas individuales de aluminio y mantenidas en una nevera de icopor con hielo hasta su utilización; esta metodología permitió reducir la oxidación del tejido foliar durante la recolección, mejorando la extracción de ADN de alto peso molecular.

Extracción de ADN

La extracción de ADN de alta calidad se logró utilizando un protocolo óptimo para especies aromáticas y/o recalcitrantes a la extracción, el cual utiliza las propiedades desnaturalizantes del catión guanidinio y la sílica (Vega-Vela y Chacón, in-press.). Básicamente, para cada muestra se maceró con nitrógeno líquido en un mortero estéril 150 mg de tejido foliar e inmediatamente se transfirió el macerado a un tubo Eppendorf de 2 mL con 800 mL de buffer previamente servido [CTAB 2% p/v, NaCl 1.5M, EDTA 20mM, Tris-HCl pH 8.0 100mM y β-mercaptoetanol 1%]. La muestra se homogeneizó por inversión y se incubó a 65°C por 30min. Posteriormente se adicionaron 800 mL de cloroformo: alcohol isoamílico (24:1) y se mezcló por inversión fuertemente hasta obtener una emulsión; esta mezcla se centrifugó a 14.400 xg durante 10 min. El sobrenadante se transfirió a un tubo Eppendorf de 2 mL nuevo y estéril, al cual se le adicionó 200 mL de isopropanol -20°C y se mezcló por inversión. Luego, se adicionó al tubo 1 mL de solución de sal de guanidina [GdmHCl 4M disuelta en agua destilada y estéril], con fuerte agitación por inversión durante 5 min. La muestra se transfirió a una columna de sílica (EconoSpin™ All-in-1 Mini Spin Columns, Epoch Biolabs) y se centrifugó a 10.000 xg durante 5 min. El flujo colectado fue descartado y la membrana se lavó dos veces con Etanol 90%. La columna se centrifugó a 10.000 xg por 10 min para secar completamente la membrana y eliminar cualquier tipo de residuo remanente. Se adicionaron 100 µL de buffer TE bajo en sal pH ≥ 8.0 precalentado a 65°C directamente al centro de la membrana, se incubó a 65°C durante 5 min y se centrifugó la columna a 10.000 xg por 1 min. El flujo colectado con el ADN se incubó con 2 µL de RNAsa A (1 µg/µL) durante 15 min a 37°C. La calidad del ADN para todas las muestras se verificó en gel de agarosa al 1%; adicionalmente, la cantidad de ADN extraído se estimó utilizando un espectrofotómetro NanoDrop™ 2000 (Thermo Fisher Scientific Inc.).

Amplificación y visualización de marcadores AFLP

Se utilizó el kit AFLP® Analysis System I (Invitrogen™) para amplificar 64 combinaciones selectivas de cebadores, siguiendo estrictamente las recomendaciones del fabricante. El análisis de los productos se realizó de acuerdo con lo recomendado por el kit, aunque la tinción del gel de poliacrilamida 6% se realizó en nitrato de plata. La evaluación se repitió dos veces para determinar la reproducibilidad de los perfiles AFLP. Cada uno de los geles obtenidos fue documentado con un escaner Epson Perfection® 4490 Photo y las imágenes fueron calificadas utilizando GIMP 2.6 y una tabla graficadora EasyPen i405

(Genius). La presencia de cada banda se resaltó utilizando una línea negra sobre la imagen mientras que la ausencia de banda se resaltó con la ausencia de color. Este procedimiento permitió la obtención confiable de la matriz binaria (presencia/ausencia) utilizando el programa Cross Checker 2.91 (Buntjer y Otsen, 1999), codificada con 1 para presencia de la banda y 0 para su ausencia.

Análisis de datos

La fase de selección se realizó utilizando una muestra de dos individuos genéticamente heterogéneos para cada una de las especies evaluadas, lo cual permitió determinar para cada una de las combinaciones de cebadores: a) número de bandas totales, b) número de bandas indiscutibles, y c) porcentaje de loci potencialmente polimorficos ($\%P_0$). Estos valores se consideraron como tres criterios mínimos para la selección de combinaciones de cebadores AFLPs en nuestro estudio. La lectura de bandas realizada en la fase inicial de selección en este estudio permitió evaluar marcadores o loci AFLP, distinto a otros estudios en los cuales únicamente se obtiene lectura de bandas o presencias, debido al número de muestras utilizadas por cada combinación AFLP. Por esta razón, el número de bandas totales se definió como el número total de loci o marcadores AFLP observados en el gel, mientras que el número de bandas indiscutibles se definió como el número de loci o marcadores que fácilmente pueden ser leídos en el gel sin asistencia de programas para la manipulación de imágenes. Similarmente, $\%P_0$ se definió como la fracción de loci o marcadores indiscutibles polimórficos (que exhiben estados diferentes: presencia/ausencia), observados en cada combinación de cebadores AFLP. Este proceso permitió seleccionar las mejores cinco combinaciones de cebadores para cada una de las especies bajo estudio.

La fase de evaluación se realizó utilizando una muestra de diez individuos por especie para cada una de las combinaciones seleccionadas. Durante este proceso se determinó: a) número de bandas indiscutibles, b) porcentaje de marcadores polimórficos por combinación de cebadores (%P), c) %P promedio por especie, y d) porcentaje de reproducibilidad del perfil AFLP para cada especie. Además, considerando que *L. origanoides* es una especie aromática promisoria por la calidad de sus aceites esenciales y usos potenciales (Celis et al., 2007; Oliveira et al., 2007; Stashenko et al., 2008), la utilidad de las cinco combinaciones de cebadores AFLP seleccionadas para esta especie se determinó mediante la capacidad de discriminación de las relaciones genéticas entre las diez accesiones utilizadas durante la evaluación, las cuales provienen de diferentes

sitios geográficos. Se calculó una matriz de distancias utilizando el método de Huff et al (1993) e implementado en el programa GenAlex 6.4 (Peakall y Smouse, 2006). Esta matriz se utilizó para construir una topología mediante el algoritmo de agrupamiento Neighbor-Joining (NJ) en el paquete Phylip (Felsenstein, 1995). La visualización de la topología NJ se realizó utilizando el programa FigTree v1.3.1 (disponible en: http://tree.bio.ed.ac.uk/software/figtree/).

Resultados

La evaluación de las 64 posibles combinaciones de cebadores del kit AFLP® Analysis System I (Invitrogen™), produjo un conjunto amplio de combinaciones que pueden ser implementadas con éxito en estudios de diversidad genética y diferenciación poblacional en las especies *L. origanoides*, *L. alba* y *T. caracasana*. En general, una gran proporción del total de las combinaciones selectivas amplificaron en las tres especies evaluadas (ver Tabla 1). Se obtuvo en promedio para *L. origanoides* 32 marcadores/combinación, 30 marcadores indiscutibles por combinación y 32%P_0. Para *L. alba*, los promedios obtenidos fueron 45 marcadores/combinación, 39 marcadores indiscutibles/combinación y 9%P_0. *T. caracasana* presentó 29 marcadores/combinación, 27 marcadores indiscutibles/combinación y 11%P_0, en promedio.

Estos resultados sugieren que pocos marcadores presentaron una lectura difícil en el gel de poliacrilamida para las tres especies, determinado principalmente por la pequeña diferencia entre los promedios de marcadores/combinación y marcadores indiscutibles/combinación (2 – 4 marcadores/combinación en promedio). Además, se observó una amplia variación entre los valores de %P_0 para cada una de las combinaciones evaluadas; *L. origanoides* presentó el mayor valor %P_0 respecto a las otras especies, posiblemente porque existe mayor heterogeneidad genética entre las muestras utilizadas de esta especie, las cuales fueron obtenidas de sitios de colecta distantes (Magdalena vs. Cañón del rio Chicamocha, COL). En un estudio similar, Yang et al (2005) recomendaron como criterio mínimo para considerar un conjunto de cebadores selectivos AFLP como candidatos para estudios posteriores, 30 bandas/combinación. En nuestro estudio, 48%, 82% y 47% de las combinaciones selectivas evaluadas cumplieron con este criterio en *L. origanoides*, *L. alba* y *T. caracasana*, respectivamente.

De las combinaciones de cebadores que cumplieron con los criterios anteriormente mencionados, se seleccionaron cinco combinaciones por especie teniendo en cuenta: i) número de bandas indiscutibles > 30 (Yang et al., 2005) y ii) porcentaje de loci potencialmente polimorficos, para su evaluación sobre una muestra de diez individuos heterogéneos genéticamente (ver combinaciones seleccionadas en la Tabla 1). La cantidad de combinaciones seleccionadas fue pequeña respecto al número posible de combinaciones que reúnen los criterios de selección; sin embargo, algunas observaciones adicionales se consideraron durante la selección, por ejemplo, la calidad de la amplificación de los fragmentos y la distribución de los fragmentos en el perfil (datos no mostrados).

Los resultados de la evaluación del grupo de cinco combinaciones por especie se observan en la Tabla 2. Básicamente, el número de bandas indiscutibles por combinación fue mayor respecto a la fase inicial para todas las combinaciones y especies evaluadas; 112, 80 y 94 bandas o marcadores por combinación en promedio, se obtuvieron para *L. origanoides*, *L. alba* y *T. caracasana*, con un aumento porcentual de 48%, 18% y 42%, respectivamente. Por otra parte, el nivel de polimorfismo obtenido en la evaluación estuvo determinado principalmente por el tipo de especie evaluada. *L. origanoides* y *T. caracasana* presentaron un aumento en el nivel de polimorfismo para cada una de las combinaciones respecto al $\%P_0$ estimado en la fase de selección. De manera contraria, *L. alba* exhibió niveles de polimorfismo similares o ligeramente inferiores respecto al $\%P_0$ estimado en cada combinación de cebadores AFLP. En promedio, 69.5%, 12.9% y 62.6% de los marcadores calificados por combinación, fueron polimórficos para *L. origanoides*, *L. alba* y *T. caracasana*, respectivamente.

Por otra parte, la reproducibilidad de los perfiles AFLP estuvo de acuerdo con lo reportado para este sistema de marcadores (Blears et al., 1998; Meudt y Clarke, 2007; Savelkoul et al., 1999); no obstante, la reproducibilidad para las combinaciones evaluadas en este estudio probablemente aumentaría al definir un rango de tamaños para la lectura de los fragmentos en el perfil, por ejemplo, 150 – 500 pb es un rango comúnmente utilizado en distintos estudios de diversidad genética. La estimación de la reproducibilidad en este reporte fue ligeramente menor respecto a lo esperado, ya que se realizó sobre la totalidad de los loci del perfil AFLP.

L. origanoides es una especie aromática promisoria por la calidad de sus aceites esenciales, amplia variación fitoquímica (Stashenko et al., 2010), bioactividad de amplio espectro sobre diferentes patógenos, fuente de fitomedicinas y usos potenciales en

diversas industrias como alimentos, cosmética, farmacológica, agrícola, etc. (Bueno-Sánchez et al., 2009; Stashenko et al., 2010). Por esta razón, se evaluó la utilidad de las combinaciones seleccionadas para el estudio de la diversidad y estructura genética en *L. origanoides*, a través de la capacidad de discriminación de las relaciones genéticas entre diez accesiones provenientes de diferentes regiones geográficas de Colombia. En la figura 1, se observa que las combinaciones seleccionadas (E-AAC / M-CTA, E-ACT / M-CAT, E-AGG / M-CAT, E-AGG / M-CTC y E-ACA / M-CAT), discriminaron claramente las relaciones entre individuos heterogéneos genéticamente por sitio de colecta; se observaron dos grupos principales, accesiones provenientes de Santa Marta (COL) y aquellas recolectadas en el Cañón del río Chicamocha (Santander y Boyacá, COL).

Discusión

En este estudio, se propuso realizar la selección rápida de combinaciones de cebadores AFLP en un solo paso, en el cual todas las 64 combinaciones posibles fueron evaluadas como mínimo en dos individuos genéticamente heterogéneos y pertenecientes a una misma especie. Esta estrategia, distinta a la realizada comúnmente durante la selección de combinaciones de cebadores AFLP (Meudt y Clarke, 2007; Yang et al., 2005), permite determinar el número de bandas total y $\%P_0$ para cada una de las posibles combinaciones selectivas de cebadores AFLP evaluadas durante el estudio. La estimación de $\%P_0$ asiste adecuadamente la selección y evita realizar pasos adicionales de evaluación – selección de combinaciones.

Considerando que el método que proponemos produce mayor información para cada una de las combinaciones, reportar estos resultados resultará conveniente durante la planificación de proyectos de investigación por parte de otros investigadores (Meudt y Clarke, 2007); por ejemplo, considerando los resultados obtenidos para *L. alba* (ver Tabla 1), diferentes combinaciones amplificaron un alto número de bandas y probablemente podrían considerarse combinaciones candidatas y apropiadas para estudios posteriores en esta especie. La utilidad de estimar $\%P_0$ para todas las combinaciones permite evitar que los investigadores inviertan recursos adicionales para evaluar combinaciones que probablemente no producirán resultados apropiados para abordar diferentes preguntas de investigación; al identificar este escenario, el investigador podría utilizar sus recursos en la exploración de nuevas combinaciones AFLP con las cuales alcance la resolución necesaria para el desarrollo de su investigación.

Yang et al (2005) reportaron una metodología para el establecimiento de marcadores AFLP utilizando 3 pasos de selección, a partir de 256 combinaciones: i) amplificación de todas las combinaciones AFLPs posibles sobre un individuo y selección de n_0 combinaciones que amplifiquen más de 30 bandas, ii) amplificación de n_0 combinaciones sobre cinco individuos y selección de n_1 combinaciones, iii) amplificación de n_1 combinaciones sobre 14 individuos y selección de n_2 combinaciones; por último, realizaron un paso de validación de las n_2 combinaciones seleccionadas (Yang et al., 2005). No obstante, aunque el esquema propuesto por Yang et al (2005) permitió obtener combinaciones adecuadas para el estudio de la variación genética en *Prunus mume*, la selección de combinaciones AFLPs utilizando esta metodología o estrategias similares, podrían resultar ser dispendiosas, costosas y demandantes de tiempo. Además, es probable que algunas de las combinaciones descartadas inicialmente, presentaran un número mayor de bandas o loci AFLP con niveles adecuados de polimorfismo si hubiesen sido amplificadas sobre al menos dos individuos heterogéneos genéticamente, en lugar de un solo individuo. A pesar que el método de selección que proponemos fue evaluado únicamente sobre 64 combinaciones, respecto a las 256 combinaciones AFLP utilizadas por Yang et al (2005), es probable que utilizando dos o tres individuos heterogéneos genéticamente durante la selección, resulte suficiente para generar la información necesaria para identificar combinaciones AFLP adecuadas sin importar el número inicial de combinaciones a evaluar.

La utilidad de las combinaciones seleccionadas para *L. origanoides* fue adecuada, ya que permitió discriminar accesiones provenientes de orígenes geográficos diferentes (Santa Marta vs. Cañón del Chicamocha, Santander y Boyacá, COL), y accesiones pertenecientes a localidades cercanas geográficamente en el cañón del río Chicamocha. Adicionalmente, las combinaciones exhibieron niveles altos de polimorfismo y reproducibilidad del perfil AFLP. Por esta razón, las combinaciones E-AAC / M-CTA, E-ACT / M-CAT, E-AGG / M-CAT, E-AGG / M-CTC y E-ACA / M-CAT son apropiadas para el análisis de la diversidad y la estructura genética de *L. origanoides*.

Conclusiones

El proceso de selección de combinaciones selectivas AFLP para el establecimiento de la técnica en especies no modelo reportado en este artículo, resultó ser adecuado para una evaluación rápida y la obtención de información crítica para tomar una buena decisión

respecto a las combinaciones selectivas de cebadores a utilizar en diversos tipos de estudios genéticos. Recomendamos realizar la evaluación inicial de las combinaciones AFLP sobre una muestra de dos o más individuos heterogéneos genéticamente por especie, estimar $\%P_0$ para cada una de las combinaciones evaluadas y seleccionar por este criterio y número de bandas.

Agradecimientos

Este trabajo fue financiado por el Ministerio de Agricultura y Desarrollo Rural (MADR) de Colombia mediante contrato No. 064-2007V7163-50-07, la Universidad Nacional de Colombia y COLCIENCIAS a través de una beca – pasantía desarrollada bajo el programa de “Jóvenes Investigadores e Innovadores” contrato No. 155-2008.

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Tabla 1. Amplificación de 64 combinaciones selectivas AFLPs en tres especies aromáticas de Colombia y promisorias por la calidad de sus aceites esenciales. Para cada una de las especies se indican el número total de bandas calificadas (Bandas), el número de bandas cuya calificación (presencia / ausencia) es indiscutible (Indiscutibles), y el porcentaje de loci potencialmente polimórficos (%P_0). El tamizaje inicial se realizó sobre dos individuos provenientes de distintos orígenes geográficos para cada una de las especies, lo cual permitió determinar las combinaciones selectivas candidatas en estudios posteriores de diversidad genética y estructura poblacional. En la tabla, se resaltan únicamente 5 combinaciones selectivas candidatas para cada una de las especies evaluadas; sin embargo, un número mayor de combinaciones cumplen con los criterios de selección, por lo cual depende del investigador y los recursos de la investigación, seleccionar el número de combinaciones a evaluar.

Combinación AFLP Invitrogen		*L. origanoides*			*L. alba*			*T. caracasana*		
		Bandas	*Indiscutibles*	*% P_0*	*Bandas*	*Indiscutibles*	*% P_0*	*Bandas*	*Indiscutibles*	*% P_0*
E-AAC [b]		53	42	33	95	81	9	53	50	4
E-AAG [b]		85	85	21	80	80	14	60	60	3
E-ACA		33	33	36	57	40	0	47	47	0
E-ACC	/ M-CAA	34	19	47	49	30	3	36	25	4
E-ACG		8	8	0	30	22	91	11	11	45
E-ACT		47	47	38	44	44	5	45	45	0
E-AGC		3	3	0	41	40	10	13	13	31
E-AGG		44	42	29	46	40	5	34	30	0
E-AAC		37	28	43	55	43	2	34	28	11
E-AAG		58	58	0	81	81	4	49	49	10
E-ACA		35	35	34	32	32	19	34	34	3
E-ACC	/ M-CAC	27	24	46	42	32	0	30	26	0
E-ACG		20	20	40	47	28	50	0	0	0
E-ACT [b]		30	30	37	54	54	24	23	20	15
E-AGC		19	19	53	35	32	6	15	13	23
E-AGG		35	32	41	47	43	5	26	24	4
E-AAC		22	19	58	32	19	0	29	23	17
E-AAG		42	42	31	47	47	4	37	37	5
E-ACA		26	26	42	30	30	7	14	14	0
E-ACC	/ M-CAG	20	20	35	21	10	0	28	18	28
E-ACG		9	9	0	65	65	38	2	2	0
E-ACT		26	23	48	41	41	2	40	40	13
E-AGC		25	25	36	31	31	3	18	16	0

E-AGG		26	24	38	42	40	5	21	20	10
E-AAC		30	0	0	52	38	0	49	39	18
E-AAG		28	28	7	56	34	15	33	33	0
E-ACA [a]		66	66	36	91	91	2	45	45	7
E-ACC	/ M-CAT	19	17	18	47	33	0	34	28	0
E-ACG		16	16	0	33	29	10	16	12	0
E-ACT [a]		48	48	31	57	41	12	48	48	6
E-AGC		31	31	39	52	52	4	30	30	7
E-AGG [a]		62	60	35	83	83	1	64	64	6
E-AAC [ac]		48	45	38	82	65	3	56	48	15
E-AAG		45	45	20	45	45	7	18	18	6
E-ACA [c]		43	43	33	40	36	6	46	38	21
E-ACC	/ M-CTA	20	16	44	29	23	0	19	15	7
E-ACG		17	17	53	31	25	4	13	13	0
E-ACT		40	40	38	62	51	4	31	31	10
E-AGC		27	26	46	47	44	2	28	27	26
E-AGG [bc]		50	50	32	64	60	8	60	60	25
E-AAC		15	15	0	34	34	0	11	11	0
E-AAG		15	15	7	56	29	28	39	39	3
E-ACA		42	42	48	52	52	0	27	27	11
E-ACC	/ M-CTC	24	22	36	32	17	6	10	9	11
E-ACG		24	24	50	21	11	9	7	7	0
E-ACT		32	32	53	69	69	6	18	18	6
E-AGC		37	25	32	42	36	0	17	15	13
E-AGG [ac]		74	74	35	51	51	6	71	71	42
E-AAC		23	23	22	6	6	0	5	5	0
E-AAG		4	4	0	7	7	0	0	0	0
E-ACA		15	10	40	18	10	0	22	14	0
E-ACC	/ M-CTG	10	9	44	6	6	0	12	5	0
E-ACG		3	3	33	5	1	0	1	0	0
E-ACT		0	0	0	10	10	0	4	4	0
E-AGC		7	4	50	4	3	33	4	4	50
E-AGG		49	49	35	37	37	0	20	20	40
E-AAC		57	57	26	93	86	1	68	56	5
E-AAG [b]		47	42	29	57	57	9	66	66	3
E-ACA		58	49	41	63	48	4	45	40	18
E-ACC	/ M-CTT	22	19	21	36	22	9	34	25	8
E-ACG		29	29	31	40	32	13	14	10	40
E-ACT		22	15	73	48	48	0	21	21	5
E-AGC		24	22	27	23	15	67	20	17	18
E-AGG [c]		56	56	38	82	82	2	53	53	38

Tabla 2. Amplificación de 5 combinaciones AFLPs candidatas para estudios de diversidad genética y estructura genética en tres especies aromáticas promisorias de Colombia. Para cada especie se indican la combinación selectiva, número de bandas indiscutibles por combinación (Indiscutibles), porcentaje de bandas polimórficas por combinación (%P/comb.) y total por especie (%P (Total)), y porcentaje de reproducibilidad de los perfiles AFLPs por especie (% Reproducib.). Note que el número de bandas indiscutibles y el nivel de polimorfismo por combinación AFLP, aumentaron significativamente respecto al tamizaje inicial (ver Tabla 1 para comparación).

Especie	***Combinación-AFLP***	***Indiscutibles***	***% P/comb.***	***% P (Total)***	***% Reproducib.***
L. origanoides	E-AAC / M-CTA	113	69.91	69.50	97
	E-ACT / M-CAT	87	67.82		
	E-AGG / M-CAT	115	75.65		
	E-AGG / M-CTC	131	69.47		
	E-ACA / M-CAT	118	64.41		
L. alba	E-AAC / M-CAA	121	14.05	12.90	98
	E-AAG / M-CAA	70	12.86		
	E-AAG / M-CTT	92	13.04		
	E-ACT / M-CAC	51	1.96		
	E-AGG / M-CTA	69	18.84		
T. caracasana	E-AGG / M-CTA	104	70.19	62.69	97
	E-AGG / M-CTC	91	86.81		
	E-AGG / M-CTT	85	62.35		
	E-ACA / M-CTA	80	42.50		
	E-AAC / M-CTA	109	50.46		

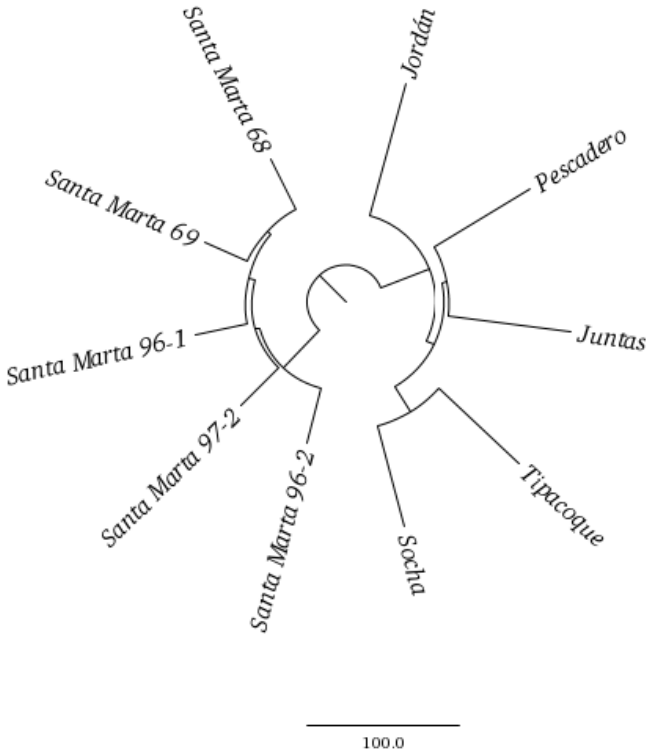

Figura 1. Relaciones genéticas entre accesiones de *L. origanoides* provenientes de dos regiones geográficas en Colombia. En la figura se observan las relaciones genéticas entre cinco accesiones provenientes de Santa Marta (Magdalena, COL) y cinco accesiones provenientes del Cañón del rio Chicamocha (Santander y Boyacá, COL). La representación radial de la topología utilizando el método de Huff et al (1993) y agrupado por Neighbor-Joining, indicó una clara diferenciación entre las accesiones provenientes de dos regiones geográficas de Colombia (Magdalena vs. Cañón del rio Chicamocha), además de una discriminación dentro de cada región.

3. Genetic diversity and Population Structure of *Lippia origanoides* from the Canyon of the Chicamocha River in Colombia.

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Abstract

Lippia origanoides is an aromatic species of the Verbenaceae family whose essential oils and extracts have shown promising effects against several kinds of microorganisms and also antioxidant activity. Despite the potential use of its essentials oils in various industries such as food, cosmetics, pharmaceutical and agricultural, very little is known about basic genetic aspects. In this study, the genetic structure of *L. origanoides* within the canyon of the Chicamocha river in Colombia and the geographic distribution of this genetic variation were evaluated using AFLP markers. The number of markers and the number of individuals sampled was sufficient to reliably determine that *L. origanoides* from the canyon of the Chicamocha river is composed of four populations found along an increasing elevation gradient: i) LBa and LBb: two populations in the lower basin, ii) MB: the medium basin population and iv) UB: the upper basin population, with a low level of admixture between them. The levels of genetic diversity within populations were relatively high (*Ht* = 0.31995; *I* = 0.4785). Nevertheless, the population UB showed the lowest genetic diversity (*hs* = 0.25096) with a reduction of twenty percent compared to the other populations, following a pattern reported for species inhabiting elevation gradients. Two-level AMOVA showed highly significant genetic differences between populations (F_{ST} = 0.17916, *p-value* < 0.0000). Demographic processes similar to isolation by distance, geographical conditions and the difference in altitude among the basins in the Chicamocha river seem to be relevant in determining the genetic structure of this species.

Keywords: AFLP; genetic similarity; isolation by distance; genetic differentiation; elevation gradients.

Introduction

Lippia origanoides H.B.K. is an aromatic shrub, erect and branched, which grows to 3 m in height and belongs to the Verbenaceae family. This shrub has simple and opposite leaves of variable sizes due to possible physiological and morphological adaptations in response to exposure to light (Parra and Rodríguez, 2007), with inflorescences characterized by white flowers, small and pedicellate (4 mm in size) (de Campos et al., 2011), and a high yield of dried fruits and seeds per plant. *L. origanoides* has a pungent odor similar to the spice known as oregano, due to the presence of secondary metabolites such as carvacrol, thymol, p-cymene, among other phenolic compounds responsible for the particular aroma and flavor of this spice (Arcila-Lozano et al., 2004; Calpouzos, 1954). Because of this, *L. origanoides* is known as “oregano de monte” and it is classified within the group of species known as “oregano”, with extracts and essential oils of comparable chemical compositions, exhibiting similar antioxidant, antimicrobial, antigenotoxic and antiparasitic activities (Arcila-Lozano et al., 2004; Calpouzos, 1954; Pascual et al., 2001). *L. origanoides* has an important phytochemical variation represented in three chemotypes according to the major compounds present in their essential oils (chemotype A: p-cymene, chemotype B: carvacrol, and chemotype C: thymol) (Stashenko et al., 2010). The bioactivity of the extracts and essential oils of these chemotypes has been evaluated with promising results in assays against *Spongospora subterranea* (Bittara et al., 2009), *Sclerotium rolfsii*, *Macrophomina phaseolina* (Ulacio et al., 2008), *Rhizoctonia solani*, *Bipolaris maydis* (Rodríguez and Sanabria, 2005), *Mycosphaerella fijiensis* (Vargas et al., 2009), *Mycobacterium tuberculosis* (Bueno-Sánchez et al., 2009), *Candida* sp. (dos Santos et al., 2004; Henao et al., 2009; Oliveira et al., 2007), *Leishmania chagasi*, *Trypanosoma cruzi* (Celis et al., 2007; Escobar et al., 2010), *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp. and others microorganisms (dos Santos et al., 2004; Henao et al., 2009; Oliveira et al., 2007; Ramírez et al., 2009). Additionally, these essential oils have showed antioxidant activity (Celis et al., 2007; Muñoz-Acevedo et al., 2009), repellent activity against *Tribolium castaneum* and *Sitophilus zeamais* (Nerio et al., 2009; Olivero-Verbel et al., 2009), antiviral effect on Dengue Virus and Yellow Fever Virus (Meneses et al., 2009a; Meneses et al., 2009b) and DNA protective effect against

bleomycin-induced genotoxicity (Vicuña et al., 2010). These results show that the extracts and essential oils of *L. origanoides* are of broad spectrum (Celis et al., 2007; Stashenko et al., 2010), with potential uses in various industries such as food, cosmetics, pharmaceutical and agricultural, and are suitable as a source of phytomedicines (Bueno-Sánchez et al., 2009; Stashenko et al., 2010; Vicuña et al., 2010).

L. origanoides has been mainly reported in northern South America (de Campos et al., 2011; Pascual et al., 2001; Suárez et al., 2008). Naturally, this species is restricted to areas with xerophytic and subxerophytic conditions (Albesiano, 2003; Albesiano and Rangel-Ch, 2006; Parra and Rodríguez, 2007), therefore it has been considered as a specialist species with high tolerance or resistance to stress imposed by environmental factors typical of the arid and semiarid environments (Antolinez-Delgado and Rodríguez, 2008; Camargo and Rodríguez, 2008; Parra and Rodríguez, 2007). In Colombia, *L. origanoides* has been reported in large semiarid areas of the departments of Santander and Boyacá (specially, in the canyon of the Chicamocha river), Cauca, Nariño and Magdalena (Albesiano and Rangel-Ch, 2006; Stashenko et al., 2010; Suárez et al., 2008). The canyon of the Chicamocha river is a semi-arid zone that encompasses an area of about 300.000 hectares that expands between the departments of Santander and Boyacá in northeastern Colombia, with elevations ranging from 300 to 2600 meters above the sea level (m.a.s.l.). In this region, *L. origanoides* is widely and abundantly distributed because of its ability to tolerate stress and its phenotypic plasticity (Antolinez-Delgado and Rodríguez, 2008; Camargo and Rodríguez, 2008; Parra and Rodríguez, 2007), and other factors such as little shepherding by goats and modification of the landscape by human activities (Albesiano and Rangel-Ch, 2006; Suárez et al., 2008).

Very little is known about basic genetic aspects of this species. In a recent study, de Campos et al (2011) reported the haploid number of *L. origanoides* as n = 12, 2n = 24. Nevertheless, additional studies are needed to establish its basic or monoploid number (x = ?) and determine the ploidy in this species (de Campos et al., 2011). On the other hand, the only study that describes aspects of genetic diversity of this species by means of molecular markers is the one of Suarez et al (2008). The authors reported the genetic diversity and spatial genetic structure in a population of *L. origanoides* from the lower basin of the Chicamocha river in Colombia. This population exhibited relatively high levels of genetic diversity (Suárez et al., 2008) similar to those reported in other species of the genus *Lippia* with similar geographical distributions and life histories (Viccini et al., 2004).

Despite its potential, basic information about the biology of *L. origanoides* has been scarce; therefore it is necessary to carry out studies on the reproductive biology of this species, the levels of inter- and intra-population genetic diversity, among others. This information will permit the development of a plant breeding program for the proper exploitation of this species and its conservation (Albesiano and Rangel-Ch, 2006; Suárez et al., 2008). For this reason, the aim of this study was to evaluate the genetic structure of *L. origanoides* within the canyon of the Chicamocha river in Colombia using AFLP-type molecular markers and the relationship of this variation with the geography of the canyon.

Materials and Methods

Sample collection

Fresh plant material for 173 individuals was collected in the canyon of the Chicamocha river between August and November of 2009. Sampling was carried out according to Suarez et al (2008). The sampling area comprised the lower, medium and upper basins of the Chicamocha river (see figure 1), in elevations that ranged from 300 m.a.s.l. to more than 2.000 m.a.s.l. For each sample, 20 to 30 young leaves were collected in zip-lock plastic bags containing silica gel and stored at -80°C until DNA extraction. For each sample collected, latitude, longitude and elevation data were recorded using a GPS from GARMIN (USA). Vouchers from each locality were deposited at the Herbario Nacional Colombiano (No. COL 550395 – 550408).

DNA extraction

High-quality DNA suitable for genetic analyses in *L. origanoides* was obtained using the chemical denaturing properties of the guanidinium cation and the silica, in a protocol optimized to aromatic and medicinal plants (Vega-Vela and Chacón, in-press.). Basically, 150 mg of dried tissue for each sample was ground in liquid nitrogen and immediately transferred into a sterile 2 mL Eppendorf tube containing 800 mL of buffer [2% CTAB w/v, 1.5 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 1% β-mercaptoethanol]. The samples were homogenized by gently inversion and incubated for 30 min at 65°C. Subsequently for each sample, 800 µL of chloroform:isoamyl alcohol (24:1) were added, mixed by vigorous shaking to achieve an emulsion and centrifuged at 14.400 xg for 10 min. The supernatant was transferred into a sterile 2 mL Eppendorf tube and 200 µL of isopropanol at -20°C were added and mixed gently by inversion. Afterwards, 1 mL of

Gdm^+-salt solution [Guanidinium Hydrochloride (GdmHCl) Solution: 4 M GdmHCl dissolved in sterile distilled water] was added and quickly mixed by inversion for 5 min. The sample was transferred to a silica-based column (EconoSpin™ All-in-1, Epoch Biolabs) and centrifuged at 10.000 xg for 5min; the flow-through was discarded. The membrane was washed twice by adding 0.5 mL of 90% ethanol and centrifuged at 10.000 xg for 5 min. In a new sterile collection tube, 100 µL of low salt TE buffer pH 8.0 preheated at 65°C were added directly in the center of the membrane and incubated for 5 min. The flow-through with the DNA was obtained by centrifugation at 10.000 xg for 1 min. Finally, 2 µL of RNase A (1 µg/µL) were added to each sample and incubated at 37°C for 15 min. The quality of each sample was checked in a 1% agarose gel. Additionally, all samples were quantified using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc.).

AFLP fingerprinting

The kit AFLP® Analysis System I (Invitrogen™) was used to amplify five selective primer combinations (EcoRI – AGG / MseI – CTC, EcoRI – ACA / MseI – CAT, EcoRI – AGG / MseI – CAT, EcoRI – ACT / MseI – CAT and EcoRI – AAC / MseI – CTA) according to preliminary results (unpublished data). The AFLP amplifications were carried out as recommended by the manufacturer. The visualization of the products was performed following the manufacturer´s instructions, although the staining of 6% PAGE was performed in silver nitrate. The gels were documented with a scanner Epson Perfection® 4490 Photo; each image was optimized and scored using GIMP 2.6 and an EasyPen i405 (Genius). This procedure of digital-scoring was performed by painting a black line on the bands indicating their presence and a white line indicating the absence of the fragment, which improved automated processing for obtaining the presence/absence or 1/0 matrix with Cross Checker 2.91 (Buntjer and Otsen, 1999) from polyacrylamide gels.

Analysis of Data

We scored only fragments in the range between 150 and 1000 bp. Two levels of polymorphism were considered for all the analyses conducted, 0.99 and 0.95 (the data set of 0.95 of polymorphism is more restrictive than the set recommend by Lynch and Milligan (1994), and no significant differences were observed. The genetic relationships among individuals were represented graphically by a PCA (Principal Coordinates Analysis) using the distance matrix under the method proposed by Huff et al (1993) based

on the algorithm of Orloci (1978) and implemented in GENALEX 6.4 (Peakall and Smouse, 2006). A 3-D PCA graphic was performed using the plot3d command of the RGL library (Adler and Murdoch, 2011) in the program R (R Development Core Team, 2011). Additionally, we used DARWIN 5 (Perrier and Jacquemoud-Collet, 2006) to calculate matrices of pairwise Dice and Jaccard dissimilarity indices. Dendrograms were constructed using hierarchical clustering by the UPGMA method (Unweighted Pair Group Method using Average). Other indices and genetic distances were checked but no significant differences were observed.

In most genetic studies, the number of markers and the number of individuals sampled are defined either arbitrarily or by available resources. However, in genetic structure studies this may have a great effect on the validity and reliability of the inferred clusters. For this reason, we used the method reported by Medina et al (2006) for determining whether the number of individuals and markers used were enough to describe the genetic structure in our sample. Basically, the SESIM code calculates for different combinations of number of individuals by number of markers, the standard error of the mean similarity index (SESIM) or distance measure by randomly subsampling matrices (Medina et al., 2006). The Jaccard similarity index and 1000 simulations were used; SESIM-values close to zero are preferred because it suggests consistency in the clustering.

We estimated the appropriate number of groups or partitions (populations or sub-populations), in addition to the level of admixture between the groups, and assigned each individual to each of the inferred populations using two Bayesian approaches implemented in the programs STRUCTURE 2.2 (Falush et al., 2007; Pritchard et al., 2000) and BAPS 5.3 (Corander and Marttinen, 2006; Corander et al., 2008a; Corander et al., 2008b).

STRUCTURE is widely used for inferring population structure, assigning individuals to populations, identifying migrants and admixed individuals. STRUCTURE implements a model-based clustering based on a Bayesian Markov Chain Monte Carlo (MCMC) approach, in which there are K populations and the individuals are assigned to these populations probabilistically. We used a model of ancestry with no admixture and a model of allele frequencies uncorrelated or independent, suitable for dominant molecular markers (Ehrich et al., 2007). Ten runs were conducted for each value of K ranging from 1 to 11, in accordance with the results observed with the graphical methods (PCA and dendrogram). The length of burnin period was one million with one million MCMC replicates after burnin. All runs were carried out at the BIOPORTAL of the University of

Oslo (www.bioportal.uio.no). Similarity among runs was estimated according to Rosenberg et al (2002), and ΔK as the mean of the absolute values of the second order rate of change of L(K) (Ln P(D) in STRUCTURE output) divided by the standard deviation according to the methodology proposed by Evanno et al (2005). Similarity and ΔK were calculated using an R-script "Structure-sum-2009" available from http://www.nhm.uio.no/ncb (Ehrich, 2006; Ehrich et al., 2007; Evanno et al., 2005; Rosenberg et al., 2002).

Similarly, we used BAPS (Bayesian Analysis of Population Structure) to infer the genetic structure in our data. Briefly, BAPS treats both K and allele frequencies as random variables, and returns one optimal partitioning and the probability of the optimal K. In order to strengthen the inferences, we used as prior the geographical coordinates of the sampled individuals. Spatial clustering and admixture of individuals based on mixture clustering were calculated using a vector of values of K from 2 to 20 and 20 replicates. Voroni tessellation and admixture clustering graphics were obtained to indicate the optimal partitioning or potential population structure and the level of admixture within the clusters inferred from the canyon of the Chicamocha river.

Genetic diversity within the clusters inferred with the methodologies described above was estimated by different methods widely applied to dominant markers. The average number of pairwise differences between individuals within clusters (Kosman, 2003) was calculated using the R-script AFLPDAT (Ehrich, 2006). By using the software AFLPSURV (Vekemans et al., 2002), the allelic frequencies were computed according to two methods: a) a square root method (Nei, 1987) and b) a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky, 1999), and the expected heterozygosity or Nei´s gene diversity (Nei, 1973) under EHW was estimated according to Lynch and Milligan (1994). A Bayesian approach implemented in HICKORY (Holsinger et al., 2002) under the full model was also used to estimate genetic diversity defined as the average panmictic heterozygosity within each population or cluster, without assuming EHW (Zhivotovsky, 1999). This software uses MCMC and implements a combination of slice sampling and a single-component Metropolis-Hastings sampler, with a Dirichlet distribution. DIC and Dbar had the smallest values in the full model, which was taken as criteria. We used the full model with the sampler parameters configured as burn-in = 20000, sample = 200000, thin = 20.

Additionally, the percentage of polymorphic loci (*P*) and Shannon´s information index (*I*) (Shannon and Weaver, 1949) was calculated using POPGENE 1.32 (Yeh and Boyle, 1997) and they were used as another measure to describe the genetic diversity.

Two approaches for determining the level of genetic differentiation among populations or clusters were used: a) θ^{II}, a measure of the amount of genetic differentiation among contemporaneous populations and estimated by HICKORY, and b) AMOVA, analysis of molecular variance among populations using pairwise difference as distance method and 100000 permutations for support, implemented in ARLEQUIN 3.5 (Excoffier and Lischer, 2010).

Results and Discussion

Sample collection

L. origanoides is an aromatic and medicinal species promising for the quality of its essential oils and the broad activity spectrum that these have shown against pathogenic microorganisms relevant to agriculture and human health (Arcila-Lozano et al., 2004; Pascual et al., 2001; Stashenko et al., 2008; Stashenko et al., 2010). In Colombia, it has been reported in semi-arid areas where specimens have been collected for the study of their phytochemical variation (Stashenko et al., 2008; Stashenko et al., 2010; Vicuña et al., 2010).

In this study, 173 samples were collected throughout the lower, medium and upper basins of the Chicamocha river (see Figure 1), where the species exhibits particularly high phytochemical variation (Stashenko et al., 2010). In the lower basin (elevation: 300 – 1000 m.a.s.l.), 73 individuals were collected at three locations (Juntas, Jordán and Pescadero – Cepitá, Santander, COL) in which previous papers suggested its existence (Albesiano, 2003; Albesiano and Rangel-Ch, 2006; Suárez et al., 2008). The greatest distance among any pair of individuals collected in this basin was 27 Km. In the medium basin (elevation: 1000 – 2000 m.a.s.l.), 66 individuals were collected from the municipalities of Capitanejo (Santander, COL) and Soatá (Boyacá, COL) according to previous reports (Albesiano, 2003; Albesiano and Rangel-Ch, 2006). The greatest distance among any pair of individuals collected in this basin was 33 Km. It was observed that the current geography within this basin does not present strong geographical barriers to gene flow because here the canyon reaches its greatest extent (see Figure 1), forming a valley or dale through which it is likely that air streams circulate over long distances, as

well as birds, insects, etc., which are likely to be important in seed dispersal. In contrast, this situation was different regarding to what was observed in the upper and lower basins, where the mountains and abrupt changes in the river probably constitute important barriers to gene flow and influence the shaping of the genetic structure of these populations (see Figure 1, areas Jordan – Cepitá, Cepitá – Capitanejo and Soatá – Socotá). In the upper basin (elevations greater than 2000 m.a.s.l.), a total of 34 individuals were collected. The greatest distance among any pair of individuals collected in this basin was 16 Km. In this basin, the abundance of *L. origanoides* was significantly lower than in the lower and medium basins, possibly because in this area sub-optimal conditions for the development of the species are found, which can be determinant in the distribution of the species. Small patches with very few individuals per patch were typically found in this area of the canyon, which was reflected in the smaller number of individuals collected.

The greatest distance among any pair of individuals in the whole sample was approximately 105 Km between the sites known as Juntas in Villanueva in the lower basin (Santander, COL) and Beteitiva in the upper basin (Boyacá, COL). The elevation range covered during the sampling was between 320 and 2595 m.a.s.l. for the entire canyon. On average, elevations for each basin were: a) 532 m. a. s. l. for the lower basin, b) 1440 m. a. s. l. for the medium basin, and c) 2350 m. a. s. l. for the upper basin.

Genetic relationships among individuals

A pairwise individual-by-individual genetic distance matrix was built following the method of Huff et al (1993) and was used to perform a 3-D PCA (Principal Coordinate Analysis using 3-D graphic). In Figure 2 it can be seen that individuals tend to group into clusters according to their geographic site of collection. Individuals from the upper basin formed a compact group and apparently are more closely related to individuals from the medium basin. This suggests that individuals collected at elevations higher than 1000 m. a. s. l. tend to share more alleles, which may be in part related to similar ecological conditions for adaptation in these kinds of environments (Byars et al., 2009). Conversely, the individuals from the lower basin formed an independent and more dispersed cluster, which agrees with greater genetic heterogeneity within this cluster. According to Suarez et al (2008), the individuals from the lower basin of the Chicamocha river, between Pescadero and Cepitá (Santander, COL), behave as a continuously distributed population. The remaining individuals that were collected in other localities from the lower basin (Juntas and Jordán,

Santander, COL) probably constitute new subpopulations, which would explain the pattern observed in the 3-D PCA.

Different indices of dissimilarity and genetic distances were calculated with similar results to those obtained in the 3-D PCA. In Figure 2b, the dendrogram calculated with the Dice's coefficient and clustered by UPGMA revealed a pattern of clustering by collection site. In this diagram, and similar to the 3-D PCA, the samples are grouped according to the basin where they were collected. It can also be observed that there seems to be low level of mixture between groups or basins, probably as a result of a discrete distribution determined by geographical barriers between populations in the canyon of the Chicamocha river. In addition, demographic processes similar to isolation by distance determined by the breeding system and seed dispersal in *L. origanoides* (Suárez et al., 2008) may be acting.

Inference of the genetic structure - Bayesian method: Choosing K.

According to Evanno et al (2005), the inference of populations in a sample, strongly influences further estimations of genetic diversity and differentiation. Graphical methods such as 2-D and 3-D PCA, dendrograms, etc., have been widely used for the determination of groups or populations on the basis of multiple indices of dissimilarity and information about geographical origin, which has been adopted as an biologically significant approach (Evanno et al., 2005; Kosman and Leonard, 2005; Krauss, 2000). However, these methods are not fully recommended because of their poor statistical support and limitations (Bonin et al., 2007; Evanno et al., 2005). For this reason, in this study the inference of the genetic structure and subsequent estimation of genetic diversity and differentiation was also done with alternative approaches to detect statistically strong or subtle signs of population genetic structure (Bonin et al., 2007).

Using the software BAPS, we found that the optimal number of partitions was K = 4 (probability = 1), with a low level of admixture between clusters or populations (see Figure 3a), suggesting that each individual belongs undoubtedly to one group or partition in which it has its ancestral source. Spatial clustering, summarized in the Vorony tessellation graphic (see Figure 3b), indicated that populations are discretely distributed throughout the space in the canyon of the Chicamocha river and that there is correspondence between the geographic location of individuals and their cluster membership. Therefore, the geographical conditions of each of the basins (for example contrasting elevations) (see Figure 3c) seem to be relevant in determining the genetic structure of this species in

the canyon, and probably other processes that depend on the reproductive system and seed dispersal of the species are also acting. However, little is known about how the species reproduces and disperses; this is a subject that deserves further research in order to understand the genetic landscape of *L. origanoides* in the canyon of the Chicamocha river (Suárez et al., 2008).

Similar results were obtained with STRUCTURE. In Figure 4, it can be seen that the modal value of the distribution of ΔK was located at K = 4, which in our data suggests that there are four groups or populations, confirming the results obtained previously with BAPS. Furthermore, in accordance with Evanno et al (2005), the value of L(K) was not a suitable criterion for determining the optimal K given that L(K) increased after reaching the real value of K in our data. The coefficient of similarity between runs carried out with STRUCTURE for K = 4 was 0.988 ± 0.0042, indicating similar population genetic structure across the different replications (see Figure 5). In Figure 6, the four groups inferred in the Bayesian analysis are discriminated in the 3-D PCA and the dendrogram calculated with the Jaccard's similarity coefficient and clustered by UPGMA. It can be observed for our data that the genetic structure is consistent across different inference methods commonly employed in studies of differentiation and genetic diversity. Additionally, the consistency of the clustering estimated with the SESIM code was appropriate for the number of individuals and markers used in this study (173 x 244; SESIM-value = 0.0194) and similarly to what was reported by Medina et al (2006), the number of markers had a great impact on the SESIM-value in our data.

It is important to consider that some areas between the populations identified in this analysis have important gaps without sampling, for instance, the non-sampled area between the municipalities of Cepitá and Capitanejo (see Figure 1). The same situation occurs between the municipalities of Socotá and Soatá in the medium and upper basins, respectively; nevertheless in this case, considering that the medium basin and upper basin populations are more related to each other, genotypes of the non-sampled area would be clustered in one of these two populations.

Genetic Diversity

Using five selective primer combinations, 355 AFLP loci were amplified and unambiguously scored using a manual scoring procedure for each allele in each locus (presence/absence of fragment) on a digital image (see Materials and Methods). A total of 244 (68.7%) and 227 (63.9%) markers were polymorphic at a level of 0.99 and 0.95,

respectively. According to Lynch and Milligan (1994), in our study the level of polymorphism suitable for the estimation of diversity and genetic differentiation would be 0.983 (3/N, where N is the number of samples) (Lynch and Milligan, 1994). However, all the analyses were performed using two sets of data configured at a level of polymorphism of 0.99 and 0.95 and no significant differences were observed, probably due to the high number of polymorphic markers (Kosman and Leonard, 2005; Krauss, 2000; Lynch and Milligan, 1994).

Table 1 shows different measures employed to quantify the genetic diversity of *L. origanoides* in the canyon of the Chicamocha river. In general, levels of genetic diversity within populations were relatively high. The populations LBa, LBb and MB exhibited similar levels of diversity across all the estimators used, varying between 0.285 and 0.319. The population UB showed the lowest genetic diversity (0.232 – 0.268) with a reduction of 20% compared to the other populations of the canyon. *L. origanoides* is considered to be a habitat specialist species, with high levels of plasticity and stress tolerance (Antolinez-Delgado and Rodríguez, 2008; Camargo and Rodríguez, 2008; Parra and Rodríguez, 2007), however, we believe that probably the sub-optimal conditions for the development of the species in the upper basin of the canyon are reflected in the lower levels of genetic diversity in this area. This scenario is consistent with the strong decline observed in the upper basin during the sampling phase with respect to the distribution and abundance of the species in the lower and medium basins, probably due to a reduction in population size by ecological factors (Byars et al., 2009).

We also used the Shannon's information index (*I*) as an alternative measure to quantify diversity (see Table 1). The populations LBa, LBb and MB showed similar levels of diversity (I = 0.44) and significantly higher than UB (I = 0.348), which is in accordance to the results presented above. Interestingly, the genetic diversity in *L. origanoides* seems to follow a pattern previously reported for species inhabiting elevation gradients, which are characterized by exhibiting a marked decrease in genetic variation in marginal populations compared to populations centrally situated (Byars et al., 2009). Taking this pattern in mind, the population UB behaves as a marginal population, in which different ecological factors have changed significantly reducing its population size and genetic diversity. In addition, in view of the fact that gene flow between populations or individuals in similar elevations is more likely than gene flow among those at different elevations (Byars et al., 2009), restricted gene flow may have been crucial in determining the current levels of genetic diversity in UB.

The individual contribution of each population to genetic diversity was determined using *Ht* (the panmictic heterozygosity in the total sample, based on mean allele frequencies), as suggested by Holsinger (1999). The contribution of each population (k) was estimated as *Ct* = (*Ht* – *Ht/k*) / *Ht* (Holsinger, 1999), where *Ht/k* is the heterozygosity calculated when removing the population k. The results show a high contribution of the population LBa to the genetic diversity (1. *Ct/LBa* = 3.27%; 2. *Ct/MB* = 0.53%; 3. *Ct/LBb* = 0.18%; 4. *Ct/UB* = -0.54%).

The effect of monomorphic markers on the estimation of the diversity was evaluated. In Table 1, the columns headed *Full loci* show the values of Nei's genetic diversity and Shannon's information index estimated using the full set of markers scored in this study, 355 AFLP loci. The estimators of the diversity were reduced in approximately 30% compared to the data sets that fulfill the parameters of polymorphism, usually 0.99 level of polymorphism.

On the other hand, the genetic diversity for the population LBb (in the municipalities of Pescadero and Cepitá) was previously reported by Suarez et al (2008) using ISSRs markers. In that study, the values of genetic diversity were *I* = 0.453 and *hs* = 0.484. Although the value of *I* reported for LBb in our study (*I* = 0.425) was similar to that obtained by Suarez et al (2008), the value of *hs* presented here is significantly different (*hs* = 0.484 vs. *hs* = 0.288), probably due to the use of different molecular marker systems (Mariette et al., 2002).

Population differentiation

The distribution of genetic diversity within and among populations in the canyon of the Chicamocha river was determined by two-level analysis of molecular variance (see Table 2). AMOVA showed highly significant genetic differences between populations (F_{ST} = 0.17916, *p-value* < 0.0000). In general, 17.9% of the variation was due to differences between populations of the canyon, while the remaining variation was due to differences between individuals within populations. The Bayesian estimate of differentiation among contemporary populations θ^{II} and implemented in HICKORY, showed a similar level of population subdivision (θ^{II} = 0.15592 ± 0.02311). Pairwise F_{ST} estimates (see Table 3), indicated relatively high differentiation among distant populations (LBA - UB, F_{ST} = 0.306), and moderate differentiation between nearby populations (LBA - LBB, F_{ST} = 0,104; LBB - MB, F_{ST} = 0.132; MB - UB, F_{ST} = 0.123).

Interestingly, the differentiation among populations decreased in 18% when the population LBa was removed and in 37% when the same procedure was performed for the population UB. This result is interesting because LBa and UB are located at the extremes of the distribution of *L. origanoides* in the canyon and at contrasting elevations (see Figure 1), and there is also a high genetic differentiation between them. Thereby, these populations (LBA and UB) may be appropriate targets for research to understand the genetic basis of high tolerance to stress in xerophytic environments and local adaptation along an altitudinal gradient.

Conclusions

In summary, with the results obtained in this study we conclude that the genetic structure of *L. origanoides* in the canyon of the Chicamocha river is related to the geography of the area (lower, medium and upper basins) and probably to the difference in altitude among the basins. The population that contributed the most to the genetic diversity was LBa, which corresponds to the municipalities of Juntas and Jordan, where further research and conservation activities should be focused. However, these previous results encourage further sampling and research in areas that have not been sampled yet in order to understand more of the adaptation and distribution of this important species in Colombia.

Acknowledgments

This work was funded by the Ministerio de Agricultura y Desarrollo Rural (MADR) of Colombia, contract No. 064-2007V7163-50-07, the Universidad Nacional de Colombia and COLCIENCIAS through a research fellowship under the “Programa de Jóvenes Investigadores e Innovadores" contract No. 155-2008.

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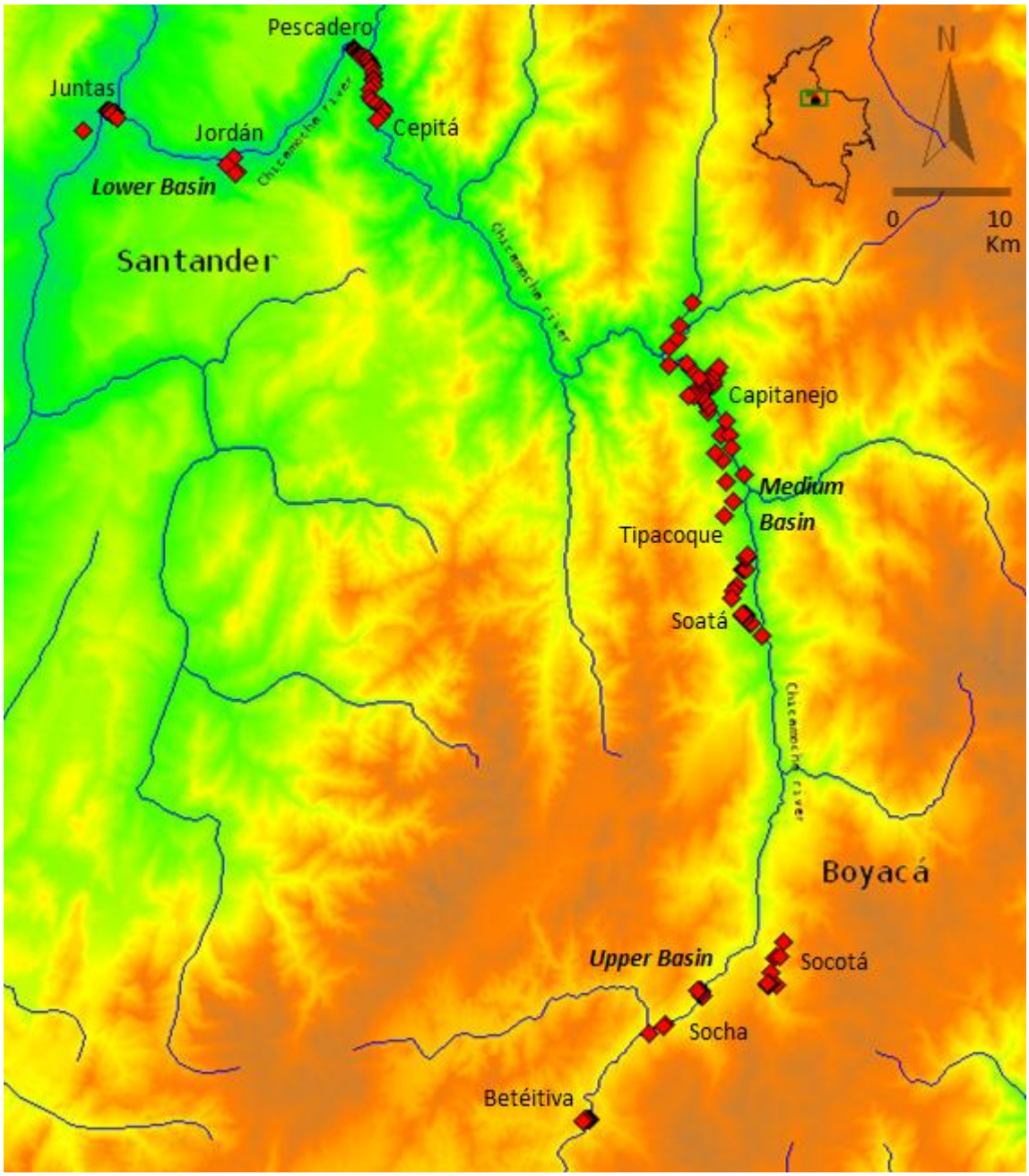

Figure 1. Map of the study area in the canyon of the Chicamocha river, indicating sampling sites. The map shows the major localities of the collection in the departments of Santander and Boyacá, Colombia. The red dots indicate the geo-referenced collection points from each individual. Samples from 73, 66 and 34 individuals were collected in the lower, medium and upper basin of the canyon, respectively.

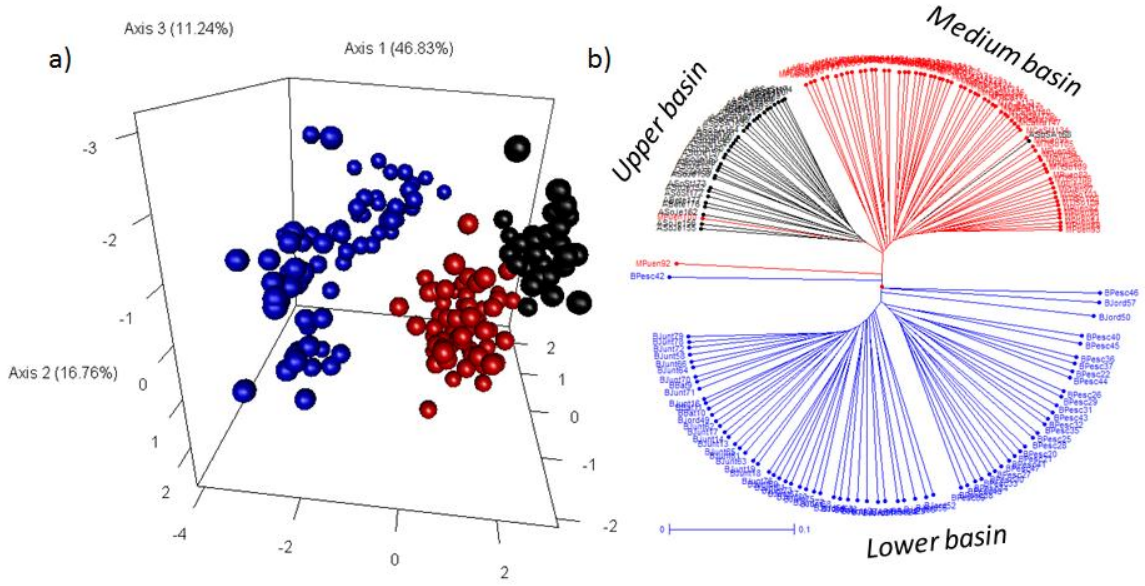

Figure 2. 3-D PCA graphic and dendrogram showing the genetic relationships of the individuals collected in the canyon of the Chicamocha river. a) The first three axes of PCA explained a 74.8% of the total variation in the data. In blue color the individuals collected in the lower basin are shown, in red color the individuals from the middle basin and in black color the individuals from the upper basin of the canyon of the Chicamocha river. The 3-D PCA used the method of Distance-Standardized implemented in GENALEX. b) The dendrogram built using Dice's coefficient and UPGMA revealed an apparent clustering of individuals from the same basin. Similar to what was observed in the 3-D PCA, the dendrogram indicated a higher genetic heterogeneity among individuals from the lower basin with respect to individuals from the medium and upper basins. In the diagrams, there is low mixture of individuals between groups, indicating a discrete distribution of populations and a clear distinction between individuals from different basins.

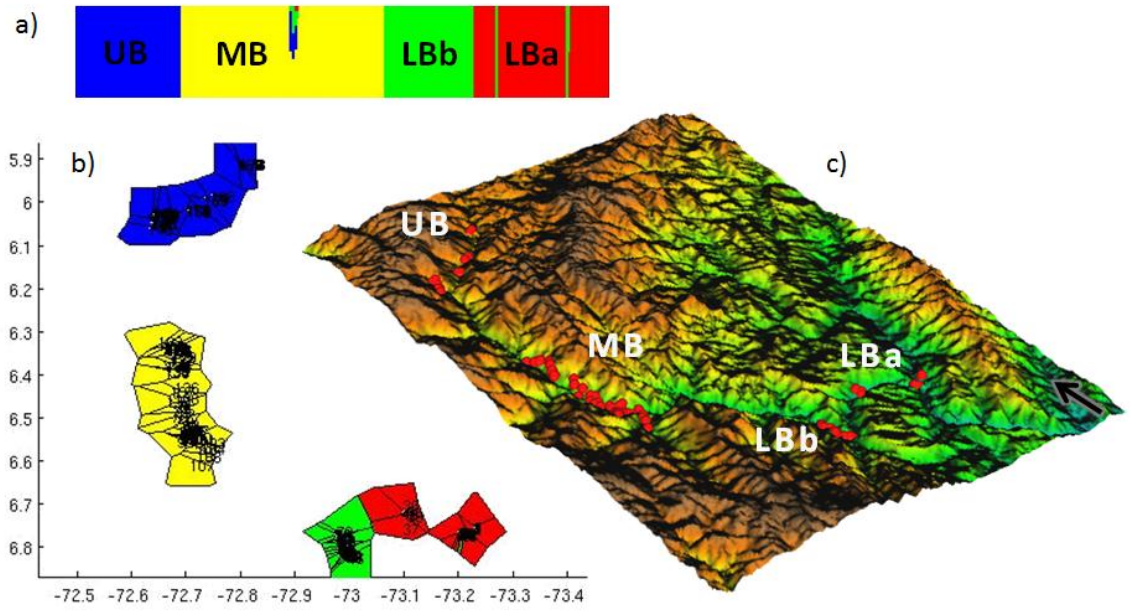

Figure 3. Admixture analysis and spatial clustering analysis using a Voroni tessellation in BAPS. Spatial clustering analysis returned an optimal partition value of K = 4, which allowed determining four populations: i) UB: population from the Upper Basin, ii) MB: population from the Medium Basin, iii) LBa: population from the Lower Basin A (Juntas and Jordán), and iv) LBb: population from the Lower Basin B (Pescadero and Cepitá). In a) Admixture analysis indicated low levels of admixture among populations. b) According to Corander et al (2008b), the spatial information increases the power to detect population structure. The Vorony tessellation graphic shows a biologically relevant scenario in which populations are distributed discretely throughout the canyon. c) A 3-D model of the Chicamocha canyon built using GRASS (GRASS Development Team, 2010) and QGIS (Quantum GIS Development Team, 2011) on the SRTM 90m Digital Elevation Data (Jarvis et al., 2006). The geographic relief of the area probably determines the distribution of the species. The model point out the populations and the individuals are indicated as red dots.

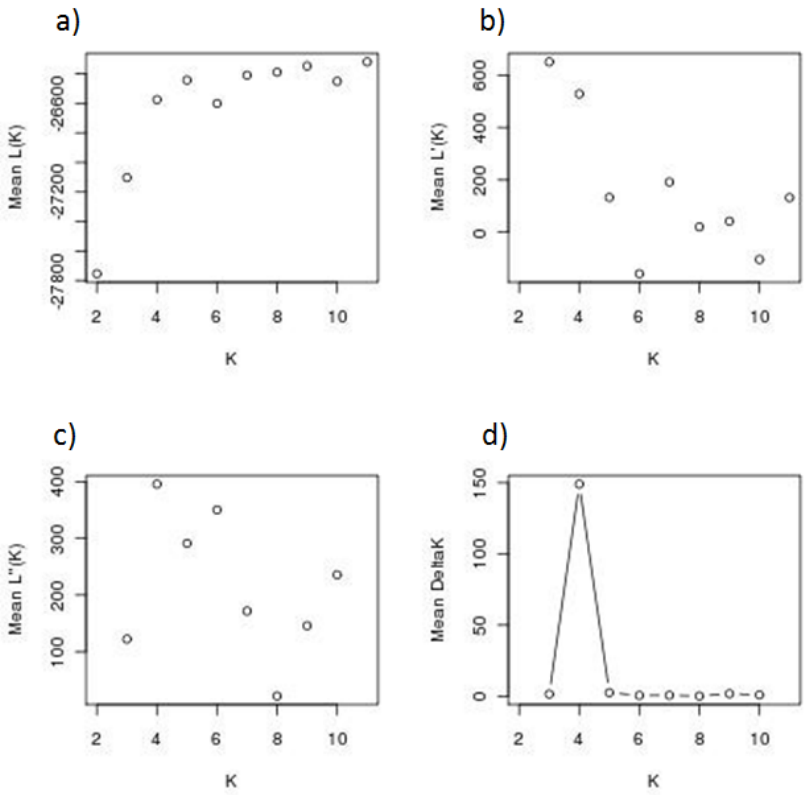

Figure 4. Population structure analysis using the methodology described by Evanno et al (2005). The figure shows the four steps for the graphical method described by Evanno et al (2005) allowing the detection of the optimal K. a) Mean L(K), over 10 runs for each K, b) Mean L'(K), rate of change of the likelihood distribution, c) Mean L''(K), absolute values of the second order rate of change of the likelihood distribution, and d) ΔK. K = 4, was the optimal K for our data. Note that L(K) increased after achieving the optimal value of K, and the height of K = 4 indicates a strong signal of genetic structure detected by STRUCTURE.

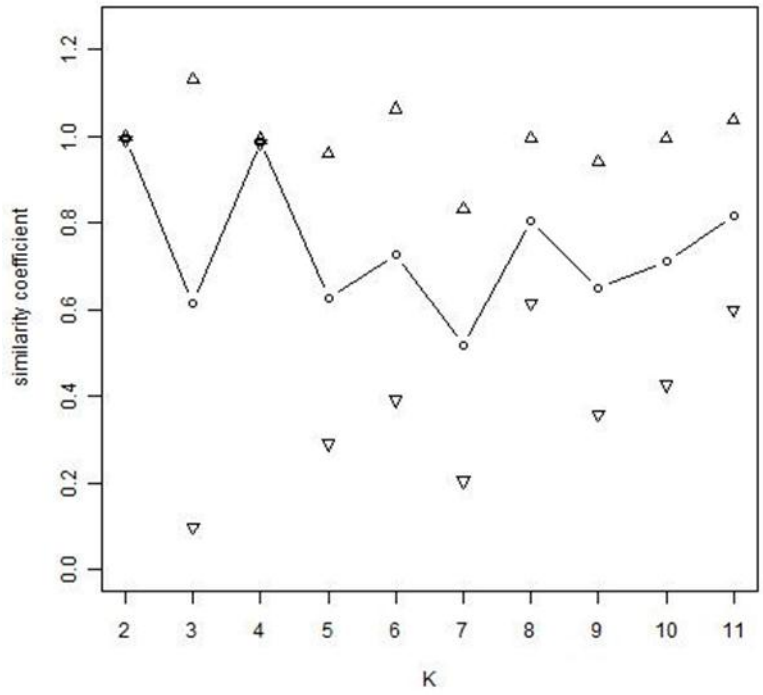

Figure 5. Similarity coefficient using the methodology proposed by Rosenberg et al (2002). The figure shows the average coefficient of similarity for each K, with standard deviation. Note that the standard deviation in K = 2 and K = 4 were smaller, indicating that the genetic structure detected by STRUCTURE was similar throughout the 10 runs.

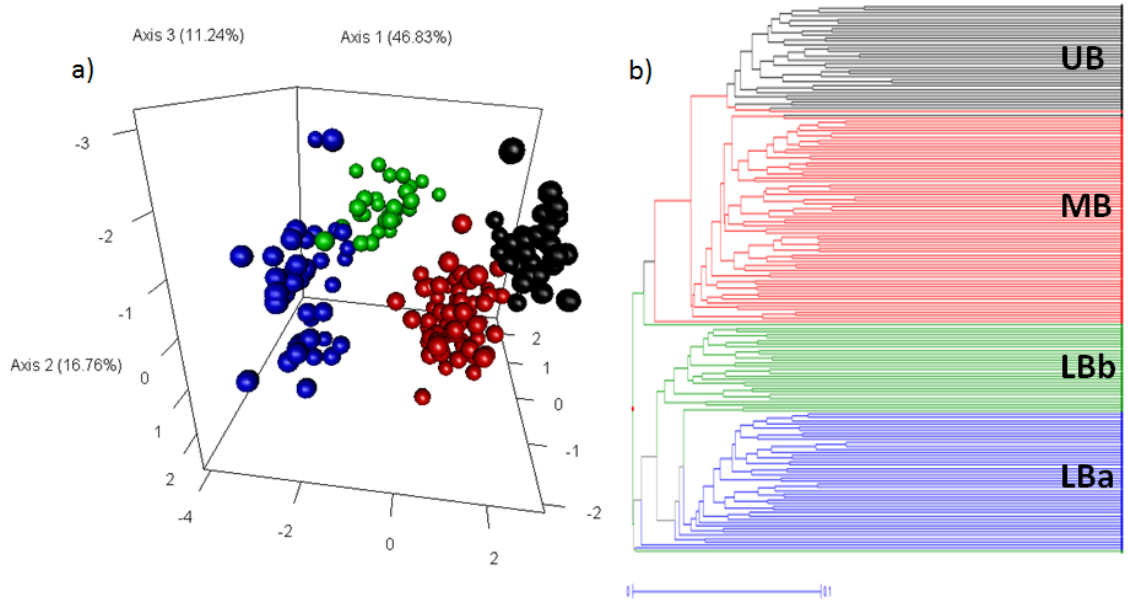

Figure 6. 3-D PCA and dendrogram showing the genetic relationships among individuals collected in the canyon of the Chicamocha river, using K = 4. a) 3-D PCA displaying the four populations identified in the Bayesian analysis. Compared to Figure 3, we found that despite having poor statistical support, the statistical graphic methods suggests the same groups or populations present in the canyon. b) Dendrogram calculated with Jaccard's coefficient and UPGMA indicating the genetic relationships among individuals and populations in the Chicamocha canyon.

Table 1. Estimates of the genetic diversity calculated by different methods.

			H (Nei)	*H (Lynch & Milligan)*				*H (Panmictic heterozygosity)*				*H (Shannon)*		*Full loci*		
Pop.	*N*	*P*	*H ‡*	*H §*	*s.d.*	*H ¶*	*s.d.*	*hs*	*Hs*	*Ht*	*s.d.*	*I*	*s.d.*	*P*	*H ‡*	*I*
LBa	44	93,03	0,28702	0,29547	0,01096	0,30806	0,01030	0,29111	-	-	0,00569	0,4419	0,21130	64,79	0,19770	0,3073
LBb	29	87,70	0,28888	0,29368	0,01222	0,30838	0,01145	0,28819	-	-	0,00446	0,4251	0,24410	60,85	0,19890	0,2949
MB	66	93,03	0,28542	0,31120	0,01098	0,31953	0,01050	0,29176	-	-	0,00732	0,4444	0,22370	64,79	0,19640	0,3086
UB	34	75,41	0,23297	0,24934	0,01279	0,26807	0,01186	0,25096	-	-	0,00810	0,3486	0,26730	52,11	0,16030	0,2409
Mean	43,25	87,30	0,27357	0,28742	0,01174	0,30101	0,01103	-	0,28051	-	0,00553	0,4150	0,23660	60,63	0,18830	0,2879
Total	173	100	0,31999	0,33710	-	0,34370	-	-	-	0,31995	0,00209	0,4785	0,18920	71,27	0,22020	0,3330

N Individuals by population
P Percentage of polymorphic loci
H ‡ Nei´s gene divesity - *AFLPdat*
H § Nei´s gene divesity using Square root method - *AFLPsurv*
H ¶ Nei´s gene divesity using a Bayesian method with non-uniform prior distrbution - *AFLPsurv*
hs Average panmictic heterozygosity within population - *Hickory*
Hs Average of hs across populations - *Hickory*
Ht Panmictic heterozygosity based on mean allele frequencies - *Hickory*
I Shannon's information index - *PopGene*

Table 2. Two-level AMOVA based on distance method of pairwise difference.

Source of variation	*d.f.*	*Sum of squares*	*Variance components*	*Fixation indices*	*P-value*	*Percentage of variation*
Among populations	3	1020,891	7,35344	0,17916	0,00000	17,92
Within populations	169	5693,837	33,69134			82,08
Total	172	6714,728	41,04478			

Table 3. Matrix of pairwise differentiation (F_{ST}: lower diagonal; *p-value*: upper diagonal) based on distance method of pairwise difference.

	LBa	LBb	MB	UB
LBa	-	0,00000	0,00000	0,00000
LBb	0,10403	-	0,00000	0,00000
MB	0,18700	0,13285	-	0,00000
UB	0,30651	0,22942	0,12302	-

4. Discusión general

En la presente investigación, se utilizaron las propiedades desnaturalizantes de la guanidina y la sílica para eliminar la alta cantidad de metabolitos secundarios presentes en las especies aromáticas, los cuales inhiben diferentes tipos de reacciones enzimáticas en laboratorio. El método descrito permitió la extracción de ADN de alta calidad a partir de un grupo heterogéneo de especies aromáticas y medicinales promisorias en Colombia, entre estas, *L. origanoides*. Adicionalmente, la evaluación de las técnicas de digestión enzimática con *MseI*, PCR y amplificación de marcadores moleculares tipo AFLP, indicaron que el ADN obtenido es adecuado para su utilización en diversas técnicas moleculares en laboratorio.

Estos resultados, además de la falta de protocolos óptimos para especies recalcitrantes a la extracción, como las especies aromáticas, y que permitan manipular fácilmente la química de la extracción, probablemente harán de nuestro procedimiento una alternativa en la investigación genética de las plantas aromáticas y medicinales en nuestro país.

Posteriormente, utilizando un único paso de selección de combinaciones selectivas de cebadores AFLP, logramos seleccionar cinco combinaciones adecuadas para la estimación de la diversidad y la estructura genética en *L. origanoides*. Estos resultados, pueden extenderse también a las otras especies evaluadas durante este estudio, *Lippia alba* y *Tagetes caracasana*, en futuros estudios de diversidad y estructura poblacional. Esta estrategia, distinto a lo utilizado generalmente durante el screening o tamizaje para seleccionar combinaciones selectivas de cebadores AFLP, utiliza además del número total de bandas por combinación, un estimador del porcentaje de loci potencialmente polimorficos de cada combinación como segundo criterio durante la selección, La estrategia, demostró que es una alternativa apropiada para obtener combinaciones de cebadores AFLP útiles en estudios poblacionales. Además, los resultados de la evaluación de las combinaciones se reportaron explícitamente, es decir, cada una de las combinaciones tiene asociada la información del número de bandas, número de bandas indiscutibles y $\%P_0$; este reporte, permitirá que otros investigadores interesados en el

estudio genético de *L. origanoides*, *L. alba* y *T. caracasana*, utilizando marcadores moleculares AFLP, puedan seleccionar un grupo de combinaciones candidato sin necesidad de realizar nuevamente el screening inicial. Lo anterior, hará que nuevas combinaciones sean evaluadas, aumentando el catálogo disponible de marcadores con utilidad en el estudio genético de estas especies.

Finalmente, se infirió la estructura poblacional de *L. origanoides* en el cañón del rio Chicamocha utilizando análisis de las relaciones genéticas entre individuos mediante coeficientes de similitud, además de métodos Bayesianos. Un total de cuatro grupos o poblaciones distribuidas discretamente a través de la geografía del cañón fueron identificados. La diversidad genética estimada en estas poblaciones fue alta, con un patrón similar al encontrado en especies distribuidas en gradientes altitudinales. La población de la cuenca alta del rio Chicamocha (UB) se comportó de manera similar a una población marginal, ya que sus niveles de diversidad fueron 20% menores en comparación con los otros grupos, además, aunque su contribución individual a la diversidad fue poco significativa, la diferenciación disminuyó cerca de un 37% al removerla del análisis. Este resultado sugirió que esta población en particular presenta genotipos distintos a los encontrados en el resto de la muestra, por lo cual es probable que procesos de adaptación local en respuesta a una altitud mayor a 2000 msnm, existan en la población UB. Por otra parte, aunque los niveles de diversidad entre las poblaciones de la cuenca baja (LBa y LBb) y la cuenca media (MB) fueron similares, la contribución individual a la diversidad de la población LBa fue significativa. Esta población al igual que la población UB, deben ser analizadas detalladamente para entender los procesos subyacentes que determinan, probablemente, la tolerancia al estrés en ambientes secos y adaptación local en respuesta a un gradiente altitudinal.

5. Conclusiones generales

El protocolo para la extracción de ADN, desarrollado en este estudio, permitió obtener cantidades y calidades suficientes para realizar cualquier técnica molecular basada en ADN sobre *L. origanoides*, además de un grupo adicional de especies promisorias con altos contenidos de aceites. Este protocolo, se espera que sea ampliamente utilizado en la investigación de especies aromáticas y medicinales, consideradas recalcitrantes a la extracción, puesto que se reportó la química del procedimiento.

La estrategia de selección de combinaciones AFLP propuesta en este estudio resultó ser una metodología rápida e informativa respecto al proceso de selección "clásico" que se ha utilizado en diversos estudios genéticos con marcadores AFLP, en los cuales el número de bandas totales es un criterio de selección suficiente para discriminar combinaciones útiles. Evaluar todas y cada una de las combinaciones en un paso inicial sobre al menos dos individuos heterogéneos genéticamente, permite estimar un segundo criterio de selección ($\%P_0$), además que reduce los pasos necesarios en la obtención de combinaciones informativas y apropiadas para estudios poblacionales.

L. origanoides está estructurada en cuatro poblaciones distribuidas discretamente a través de la geografía y el gradiente altitudinal del cañón del río Chicamocha; Similar a otras especies distribuidas en gradientes altitudinales, la población UB en la cuenca alta del rio fue considerada marginal debido a su importante disminución en la diversidad genética. Por otra parte, la población LBa de la cuenca baja del rio presentó la mayor contribución a la diversidad de la especie en el cañón. Por esta razón, es necesario adelantar investigaciones adicionales en las poblaciones LBa y UB que permitan entender los procesos de diferenciación adaptativa en respuesta a un gradiente altitudinal y la alta tolerancia al estrés impuesto por los ambientes áridos, típicos del cañón del rio Chicamocha.