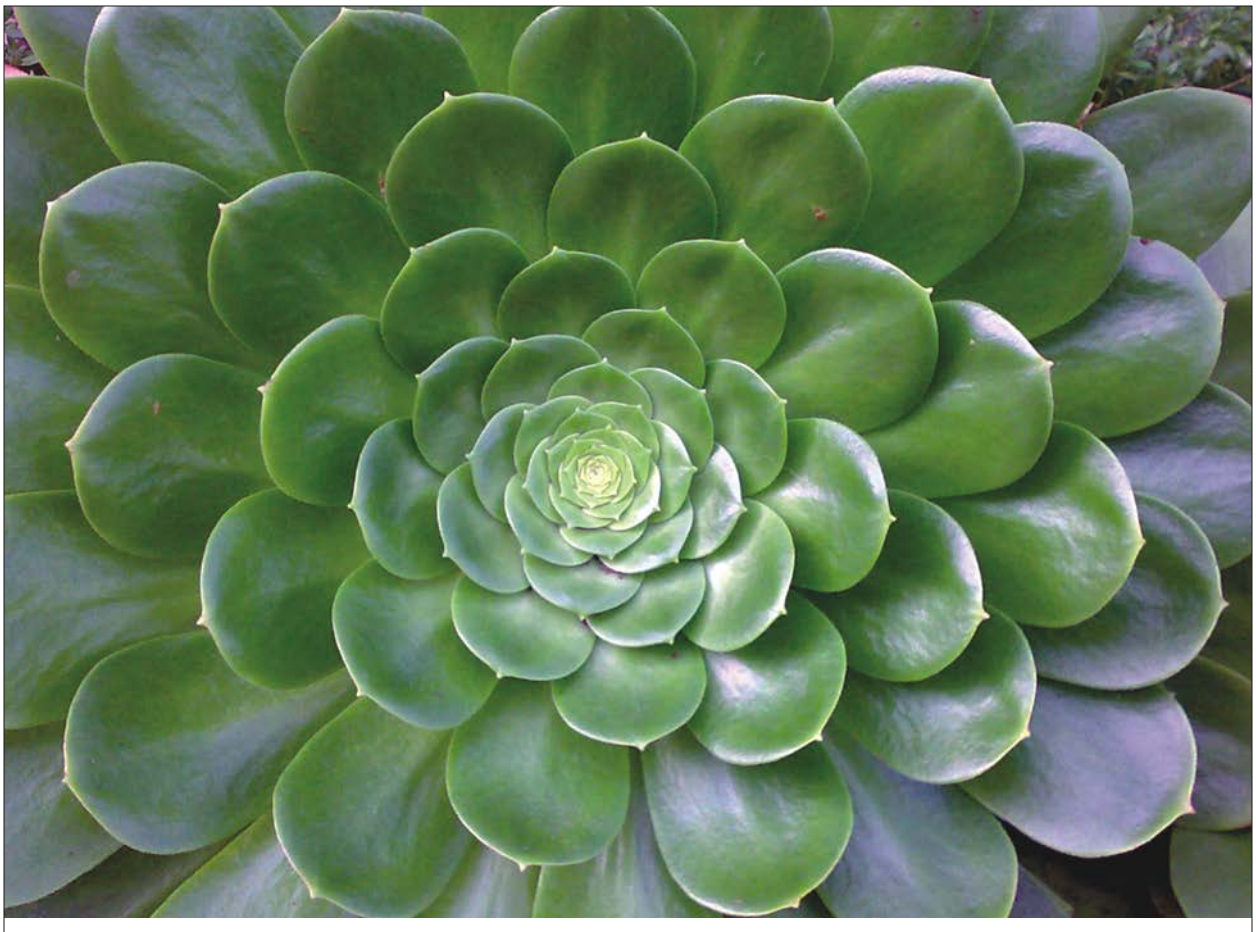

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DNA Barcoding: An Alternative for the Identification of the Medicinal Plants Employed in Mexico

[1]Jaime Marcial-Quino, [2]José Alberto Mendoza-Espinoza and [2]Edgar Sierra-Palacios
[1]Researcher from Cátedras CONACyT Commissioner to the Instituto Nacional De Pediatría, Secretaria de Salud, México D.F. 04530, México
[2]Colegio de Cienciasy Humanidades, Plantel Casa Libertad, Universidad Autónoma de la Ciudad De México, México D.F. 09620, México

Corresponding Authors: Edgar Sierra-Palacios and José Alberto Mendoza Espinoza, Colegio de Cienciasy Humanidades, Plantel Casa Libertad, Universidad Autónoma de la Ciudad de México, México D.F. 09620, México Tel: +52 55 58580538 Fax: +52 55 58321707

ABSTRACT

The use of plants for the treatment of illness is a common practice in Mexico. Almost 80% of the population utilizes medicinal plants at some point of their lives. The high demand of these plants generates the production of a diversity of herbal products in some of which different vegetal species are used as adulterants that can generate non-desired effects. This problem has led to the search of different methods for the identification of the vegetal species used in the herbal preparations. However, the path has not been obstacle free, some of these methods requires specific characteristics of the analyzing material, others shows low reproducibility and high costs. In this context, the present review covers diverse aspects of the identification methods in particular, the DNA barcoding. This a simple, low cost methodology used to identify medicinal plant materials in various presentations. The implementation of this methodology for the quality control of medicinal plants products in Mexico, would bring a number of benefits both, the herbal industry and the consumers, since the authentication of medicinal plants by DNA barcoding will guarantee high quality herbal medicinal products, without adulterants and without side effects, which would have a direct impact on users health, in addition to detonate the growth of the national herbalist sector.

Key words: DNA barcoding, molecular markers, Mexican medicinal plants

INTRODUCTION

During the history of mankind, man has made use of diverse plants for their benefit, from the recollection, as food up to the treatment of diseases. Different cultures took these practices and by tradition became subsequently of everyday and even related to religious rites, surprisingly getting benefits on the health of the communities.

The World Health Organization (WHO) reports that in many emerging countries such as Mexico and Indian around 80% of the population use medicinal plants for the treatment of various diseases. It defines a medicinal plant, as any plant species that contain compounds that can be used for therapeutic purposes or whose active ingredients are used, as precursors for the synthesis of new drugs (WHO., 1979). Due to the high consumption of herbal products, different countries have adopted. This medicinal practice as an important income source, only in China earnings were 14,000 million USD in 2005 while in Brazil in 2007 revenues earned from the sale of herbal products were 160 million USD (WHO., 2008).

In Mexico, the use of medicinal plants by pre-Hispanic cultures was not an exception. All the cultures that flourished in Mexico, only to mention the Mayan, Zapotec, Nahua, Olmec, Totonac, etc., used medicinal plants for the treatment of gastrointestinal, dermatological, respiratory, ophthalmic and gynecological diseases, for the treatment of poisoning and the bites or stings by poisonous animals (Heinrich *et al*., 1998). Today, for the Mexican population herbal medicine is more than a resource, it is a real need for the treatment of various medical conditions such as diabetes, obesity, cancer etc. (Lozoya, 1994).

Mexico has a wide variety of medicinal plants: it is estimated that Mexican medicinal flora contains between 3,000 and 5,000 plants species with therapeutic potential, although, other authors comment that the vegetal richness could reach 30,000 plants, which places Mexico as the second country with the highest number of medicinal species, after China (Lozoya, 1994). Around 3,000 species have been reported as used by the Mexican population. According to the Latin-American Society of Natural and Traditional Medicine, the Mexican herbal industry sells about 1, 200 t month^{-1}, of which the Sonora market of Mexico City, participates with approximately 160 t.

The continuous use for new herbal products with health benefits for the society, causes the launching into the market of products containing unreliable plant species and high costs but can also can be generate several type of secondary effects in the consumer. This is the reason, why new techniques are required in order to identify possible contaminants or adulterants (plants contained in the product in addition to or as a substitute for the originally intended medicinal ones). Currently molecular tools are known that are used for the identification of polluting species of commercial products and can be proposed as an alternative for the quality control of plant preparations (Hao *et al*., 2010a; Chen *et al*., 2010).

The following review aims to describe an alternative that has been used for the identification of medicinal plants in other parts of the world other than Mexico. The implementation of this methodology in the process of quality control of medicinal plant materials in our country would lead to the implementation of a stricter sanitary standards for these products, which would have a direct impact on users health, in addition to detonate the growth of the national herbalist sector.

Conventional methods for the authentication of medicinal plants: Quality control can be achieved by the combination of different techniques, from morphological and microscopic observations in order to identify medicinal plants at the species level, however, skilled personnel is required to perform these analyses. In the case of phytochemical analysis with techniques as TLC: Thin layer chromatography, HPLC: High pressure liquid chromatography, IR: infrared spectroscopy, NMR: Nuclear Magnetic Resonance and X-ray, restrictions arise from the complexity of analyses the fragmented or powdered materials, which do not allow full identification (Mukherjee *et al*., 2010).

Therefore, has been development several methods of species identification that allow to be applied to a large number of medicinal plants (Smillie and Khan, 2010). In the 1990 decade, the researchers began to work with the design of certification methods using the genome of medicinal plants (Shaw *et al*., 2002; Techen *et al*., 2004; Sucher and Carles, 2008; Hao *et al*., 2009a). These methods favored the DNA polymerase (PCR) technique and the use of different DNA thermostable polymerases. Today, molecular techniques are powerful tools for DNA fingerprinting for sample identification of medicinal plants (CBOL Plant Working Group, 2009; Hao *et al*., 2009b; Chen *et al*., 2010).

Some molecular techniques currently employed for the identification of medicinal plants are amplified fragment length polymorphism (Passinho-Soares *et al.,* 2006), Restriction Fragment

Length Polymorphism (RFLP) (Diao *et al*., 2009), random amplification of polymorphic DNA (Cao *et al*., 2010), Inter-simple sequence repeat (Tamhankar *et al*., 2009), Simple Sequence Repeat (SSR) (Sharma *et al*., 2008) and through microarray design (Carles *et al*., 2005; Jayasinghe *et al*., 2007; Zhu *et al*., 2008), which determine the variations of nucleotides in a DNA sequence belonging to the genome of plants, in particular of conserved sequences of genes present in the nucleus, mitochondria or chloroplast (Hao *et al*., 2008, 2010a). These genomic fingerprints can differentiate between genera and species, demonstrating its usefulness for characterizing plants of medicinal interest and in consequence, the detection of possible adulterants. The identification and certification of medicinal plants using specific sequences of DNA is a work in progress which offers new possibilities as a measure for the assurance of the quality of these products. Currently, there is an abundant research of certification methods that provide accurate information, are reproducible, can be automated and are developed in a short time, with a low cost. In this way, molecular methods can become a feasible alternative for the certification and quality control of medicinal plants.

Another molecular method, which has led to the rapid, accurate and automatic identification of plants, using DNA sequences, is called DNA barcoding, the main characteristic of this method is the use of short, standard and unique sequences of the genomic DNA for the identification of botanical species (Hao *et al*., 2008, 2009a, 2010b; Gao *et al*., 2010; Chen *et al*., 2010; Gu *et al*., 2013). The technique, identifies an unknown sample by comparison with a known reference plant material, its main application includes the identification of plants using fragments of leaves, when other plant organs are not available, as well as, the identification of individual species in mixtures. For these reasons, they can be used for the identification of commercial products, for example, herbal products (Kress *et al*., 2005; Seberg and Petersen, 2009).

Gene regions and employed oligonucleotides in DNA barcoding for the identification of medicinal plants: No universal consensus exists on which should be the sequence or genetic marker of DNA to be used for the identification of plants, different genomic regions have been analyzed in order for them to be considered "genetic fingerprints" (Hao *et al*., 2009b). In Table 1, some the regions used are mentioned, in the nucleus, mitochondria, chloroplast and other organelles, which have been analyzed and reported, as possible barcoding.

The data shown in Table 1 indicate that the chloroplast gene sequences have been the most explored, as possible barcodes and represent a good prospect for this technique due to the fact that many of these genes are highly conserved regions at their ends but with variability of bases inside, allowing to differentiate sequences between species. In 2009, the plants of the CBOL workgroup proposed two coding regions of the genome of the chloroplast *rbcL* and *matK* as bar codes for identification of plant species while the group of plants from China (China Plant BOL Group-CPBG) proposed the nuclear regions ITS-ITS2 as the DNA barcode for the identification of

Table 1: Genomes of three employed organelles as candidate barcodes

Mitochondria	Chloroplast	Nucleus
CO1 (Kress *et al*., 2005, 2007; Chase *et al*., 2005)	*accD* (Hao *et al*., 2010b)	ITS1
	*ndh*J (Hao *et al*., 2010b)	ITS 2 (Hao *et al*., 2010b)
	rpoB (Hao *et al*., 2010b)	ITS (ITS1-ITS2)
	matK (Lahaye *et al*., 2008; Guo *et al*., 2011)	5.8 S rDNA (Kress *et al*., 2005; Kress and
	rbcL (Guo *et al*., 2011)	Erickson, 2007; Chase *et al*., 2005)
	rpoC1 (Kress *et al*., 2005)	18S rDNA (Zhu *et al*., 2008)
	psbA-trnH (Kress and Erickson, 2007; Guo *et al*., 2011)	26S rDNA (Tsoi *et al*., 2003)
	ycf5 (Chen *et al*., 2010)	

plants from seeds (Li *et al*., 2011a; Hollingsworth, 2011). These reports show a significant progress in the identification of plants, particularly the medicinal ones, consolidating itself as a good molecular detection method.

The DNA barcodes (matK, *rbc*L, *psb*A -*trn*H, ITS, ITS2, *rpo*C1, *ycf*5, etc.) that have been tested for the identification of plant species are many, which has permitted the design of a large number of genes or spacer regions oligonucleotides, allowing to identify plants at the family level, gender or species (Song *et al*., 2009; Chen *et al*., 2010; Shi *et al*., 2011). Oligonucleotides reported for the identification of different species of plants are listed in Table 2.

Table 2: Oligonucleotides designed and used as molecular markers as possible barcodes

Markers	Primer names	Primer sequence (5'®3')	References
accD	accD 1	AGTATGGGATCCGTAGTAGG	Ford *et al*. (2009)
	accD 2	GGRGCACGTATGCAAGAAGG	
	accD 3	TTTAAAGGATTACGTGGTAC	
	accD 4	TCTTTTACCCGCAAATGCAAT	
ITS	ITS3	GCATCGATGAAGAACGCAGC	White *et al*. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	Chen *et al*. (2010)
	5s fwd	CCTTATCATTTAGAGGAAGGAG	Stanford *et al*. (2000)
	4 rev	TCCTCCGCTTATTGATATGC	White *et al*. (1990)
ITS2	S2F	ATGCGATACTTGGTGTGAAT	Chiou *et al*. (2007)
	S3R	GACGCTTCTCCAGACTACAAT	
rbcL	1f	ATGTCACCACAAACAGAAAC	Lledo *et al*. (1998)
	724r	TCGCATGTACCTGCAGTAGC	
	*rbc*L_1	ATGTCACCACAAACAGAAACT	Guo *et al*. (2011)
	*rbc*L_991	CGGTACCAGCGTGAATATGAT	
atpB-rbcL	S2r	AGAAGTAGTAGGATTGATTCTCATA	Hoot *et al*. (1995)
	RBCL1	GAATCCAACACTTGCTTTAGTCTCT	
atpF-atpH	atpF	ACTCGCACACACTCCCTTTCC	Hollingsworth *et al*. (2009)
	atpH	GCTTTTATGGAAGCTTTAACAAT	
psbA-tnrH	psbA3'f	GTTATGCATGAACGTAATGCTC	Sang *et al*. (1997) and Seberg
	trnHf	CGCGCATGGTGGATTCACAATCC	and Petersen (2009)
	psbA3	GTTATGCATGAACGTAATGCTC	Guo *et al*. (2011)
	trnH05	CGCGCATGGTGGATTCACAATCC	
tnrL-F	e	GGTTCAAGTCCCTCTATCCC	Taberlet *et al*. (1991)
	f	ATTTGAACTGGTGACACGAG	
psbM-trnD	psbM1	GCGGTAGGAACTAGAATAAATAG	Lee and Wen (2004)
	trnD	GGGATTGTAGTTCAATTGGT	Hoot *et al*. (1995)
trnK-rps16	trnK5'r	TACTCTACCRTTGAGTTAGCAAC	Johnson and Soltis (1995)
	rps164547mod	AAAGGKGCTCAACCTACARGAAC	
	trnC	CCAGTTCAAATCTGGGTGTC	Johnson and Soltis (1995)
	petN1r	CCCAAGCAAGACTTACTATATCC	
trnc-ycf6	390F	CGATCTATTCATTCAATATTTC	Demesure *et al*. (1995)
	1326R	TCTAGCACACGAAAGTCGAAGT	Lee and Wen (2004)
matK	2.1	CCTATCCATCTGGAAATCTTAG	Cuenoud *et al*. (2002)
	5-R	GTTCTAGCACAAGAAAGTCG	
rpoC1	rpoC1 1(f)	GTGGATACACTTCTTGATAATGG	Guo *et al*. (2011)
	rpoC1 2 (f)	GGCAAAGAGGGAAGATTTCG	
	rpoC1 3 (r)	TGAGAAAACATAAGTAAACGGGC	
	rpoC1 4 (r)	CCATAAGCATATCTTGAGTTGG	
rpoB	rpoB1	AAGTGCATTGTTGGAACTGG	Ford *et al*. (2009)
	rpoB1	ATGCAACGTCAAGCAGTTCC	
	rpoB1	CCGTATGTGAAAAGAAGTATA	
	rpoB1	GATCCCAGCATCACAATTCC	
ycf5	ycf5 1	GGATTATTAGTCACTCGTTGG	Ford *et al*. (2009)
	ycf5 2	ACTTTAGAGCATATATTAACTC	Ford *et al*. (2009)
	ycf5 3	ACTTACGTGCATCATTAACCA	
	ycf5 4	CCCAATACCATCATACTTAC	
ndhJ	ndhJ 1	CATAGATCTTTGGGCTTYGA	Schmitz-Linneweber *et al*. (2001)
	ndhJ 2	TTGGGCTTCGATTACCAAGG	
	ndhJ 3	ATAATCCTTACGTAAGGGCC	
	ndhJ 4	TCAATGAGCATCTTGTATTTC	

The search for the DNA barcode has been focused on genes from the chloroplast genome, since many of these genes contain highly conserved regions and serve effectively to the identification of plants. Currently, the genome of 77 of chloroplast in species is known and 789 nucleotide sequences of genes are reported. The function of the majority of these sequences is unknown but for the purpose of taxonomic identification, they have been observed to have highly conserved Table 1 (chloroplast.ocean.washington.edu/). The existence of these sequences would facilitate the elaboration of different oligonucleotides which would be useful in the identification of plants for example only the gene *acc*D (76 sequences), *ndh*J 76 of *rpo*B 111, *mat*K 72, *rbcl* 102, *rpoc*1 103, *psb*A 104, *trn*H 94, *ycf*5 44 (Cui *et al*., 2006; Nock *et al*., 2011).

The efficiency of the reported oligonucleotides (Table 2) has been proven in various studies. Song *et al*. (2009) and Hao *et al*. (2009a, b, 2010b) were among the first to use DNA barcoding for the identification of medicinal plants, using 8 DNA markers as candidates to be used as "Barcode", 7 of them in chloroplast and only one of the nucleus. The genes analyzed by these authors were *acc*D, *rbcL*, *psbA-plastid*, *ndhJ*, *rpoB*, *rpoC1*, *ACCD*, *YCF5* and *nrITS*. They found that the 6 mentioned first showed 100% efficiency of amplification for fragments of the desired genes, while *ycf*5 only 56% and *nr*ITS 44% of efficiency. These results allowed the authors to differentiate between one species and another.

On the other hand, the plant group of the consortium of the "Code Bars Of Life" (CBOL) as mentioned before utilized the regions *rbc*L and *mat*K as possible "barcode", both from the chloroplast genome. They found that the use of these single locus allowed them to discriminate against 72% of plant species, from 907 samples of 550 species (CBOL Plant Working Group, 2009). The results obtained from these samples showed that the two locus utilized for the barcoding are far from being the ideal markers for this technique, due to their low rate of identification.

The spacer present also in the chloroplast *psbA-plastid* region has been the most widely used in recent years for the identification of plant species. The spacer region obtained percentages of discrimination of 92.1% in Gymnosperms (Yao *et al*., 2010) and a 94.1% of efficiency of amplification for medicinal plants like the *Pteridophytes* (Ma *et al*., 2010). In 2010, Chen and colleagues used 7 DNA markers as candidates for DNA barcoding-*psbA-plastid matK*, *rbcL*, *rpoC1*, *ycf5*, *ITS2* and *ITS*-medicinal plant species, finding that the internal transcript of the second spacer (ITS2) of the nuclear rDNA is the most suitable region for applications of this technique. In another analysis, Chen *et al*. (2010) tested the capacity of discrimination of ITS2 in more than 6,600 samples of plants belonging to 4,800 species of 753 different genera and found that the rate of identification with the ITS2 was 92,7% at the level of species. Thus, the ITS2 region can potentially be used as a "barcode" of the standard DNA to identify medicinal plants and its closely related species. Moreover, the ITS2 could serve as a new universal bar code for the identification of a wide range of plant taxa, since when they analyzed dicotyledons monocotyledons, gymnosperms, ferns and mosses, they obtained identification rates of 76.1, 74.2, 74.2, 88.1 and 77.4%, respectively (Yao *et al*., 2010). On the other hand, Gao *et al*. (2010) demonstrated that the ITS2 sequences are adequate method to identify at the level of genus and species within the family Fabaceae, reporting that this marker has worked adequately, when used in testing large-scale, obtaining identity percentages of 90% at the species level and 100% at the level of genus.

The progress shown by these authors while using specific genes or spacer regions as possible barcoding, makes this technique a powerful tool as compared to other methods for the taxonomic classification of species; however, this technique requires two basic factors in order to obtain high

levels of identification: first, that there is a difference in the obtained fragment length and second, that these fragments have nucleotide differences which allows a differentiation between some similar species (Hao *et al*., 2010b).

Several efforts have been made in order to achieve a possible consensus regarding the regions to be used for the identification of plants. However, one of the problems that arise when molecular markers are used is that when there are phylogenetically close species in a mixture, the obtained PCR products are of a similar size, which complicates their identification.

DNA barcoding as a proposal for the authentication of medicinal plants used in Mexico: The interest of Mexicans to know the therapeutic properties of medicinal plants is due to the need to treat infections, illnesses, injuries and other cultural conditions. Since before the arrival of the Spanish colonists in Mexico, the study of genetic plant resources had already begun, as can be deduced from the development of botanical gardens. Therefore, there is an important tradition of studies on Mexican medicinal plants, even though this effort is clearly insufficient given the great diversity of medicinal plants within our country (Bye, 1995).

The use of herbs in Mexico is regionalized, being the central and southern areas the greatest consumers of medicinal plants, followed by the west, northeast and north. Although, there are no specific state or regional studies, some figures are known. In the west, north and south of the country there are no detailed reports on species marketed and used, only data on the most widely used medicinal plants, which are consistent with other reports from other parts of Mexico (Juarez-Rosete *et al*., 2013). In the case of the Northeast, Gonzalez-Stuart (2010) conducted a study in the city of Monterrey, Nuevo León, finding that about 56 different medicinal plants from 27 botanical families are employed while in the Sonora market in Mexico City, the greatest site for medicinal plants trade nationwide, 10 t of medicinal plants are marketed daily, corresponding to about 250 species.

More detailed reports on the use of medicinal plants nationwide, classify plants according to the medical conditions for which they are employed, these 220 species are used for the treatment of gastrointestinal disorders, 141 for dermatological conditions and injuries, 115 for genitourinary diseases and reproductive biology, 81 for the respiratory system, 54 for disorders of the nervous and cardiovascular system, more than 42 of ritual or magical use. In total, 653 botanical varieties are used that are widespread across the country. The Plants of greater use in the Mexican Republic are Basil (*Ocimum basilicum* L.), Arnica (*Heteroteca* inuloides Cass). The Peru Balsam (*Myroxylon balsamum* L.), Cuachalalate (*Juliana adstringens* Schltdl), Damiana (*Turnera diffusa* Willd), Epazote (*Chenopodium ambrosioides* L.), Flor de Azahar (*Citrus sinensis* Osbeck) Flower of manita (*Chiranthodendron pentadactylon* Larreat). Mullein (*Gnaphaliumo xyphyllum* DC). Master herb (*Artemisia mexicana* Wild), Hibiscus flower (*Hibiscus sabdariffa* L), Laurel leaf (*Litsea glaucescens* H. B. K), Chamomile (*Matricaria recutita* L), Mint (*Mentha spicata* L), Eye of deer (*Mucuna pruriens* DC), Pericón (*Tagetes lucida* CAV.), rue (*Ruta graveolens* L.), Aloe Vera (*Aloe vera* Burm.), Santa Maria (*Chrysanthemum parthenium* Bernh), Flor de Sauco (*Sambucus mexicana* Presl.), Tila flower (*Tilia vulgaris* Haine.), Melissa (*Agastache mexicana* (Kunth) Lint and Epling), valerian (*Valeriana officinalis* L.) and sarsaparilla (*Smilax aspera* L.) (Juarez-Rosete *et al*., 2013).

It is noteworthy that these plants highly used in Mexico, have been studied in other regions of the world using some DNA barcoding markers (Table 3), which shows that quality checks and authenticity testing can be performed by using this same methodology in Mexico.

Table 3: Identification with DNA barcoding of some medicinal plants in the world that have a high demand in Mexico

Medicinal plants	DNA barcoding markers used	Country of study	Bibliographic references
Ocimum Basilicum L	*mat*K; *Rbc*L y *trn*H-*psb*A	Italy and China	De Mattia *et al*. (2011), Suhua *et al*. (2003), Kaufmann and Wink (1994) and Christina and Annamalai (2014)
Myroxylon balsamum (L.) Harms	*mat*K; RbcL y ITS2	USA; Brazil; Germany and Mexico	Doyle *et al*. (1997) and Bold Systems (2015)
Turnera diffusa Willd	matK; RbcL y ITS2	Costa Rica and Canada	Bold Systems (2015)
Citrus sinensis	matK; RbcL y ITS2	Japan, China, USA and India	Nalumpang *et al*. (2002), Penjor *et al*. (2010) and Bold Systems (2015)
Chiranthodendron pentadactylon Lam	matK	USA	Nyffeler *et al*. (2005)
Hibiscus sabdariffa Linn.	ITS2	USA	Bold Systems (2015)
Matricaria recutita L.	matK y RbcL	UK	De Vere *et al*. (2012)
Mentha spicata L	matK, RbcL y ITS2	Germany; Italy; USA, China, Greece y Turkey	De Mattia *et al*. (2011), Brauchler *et al*. (2010) and Bold Systems (2015)
Mucuna pruriens L.	matK	Thailand	Wiriyakarun *et al*. (2013)
Tagetes lucida Cav.	ITS2	Mexico	Bold Systems (2015)
Ruta graveolens L.	matK; RbcL y ITS2	Austria, Switzerland, Australia, France, India and China	Muellner *et al*. (2003), Gadek *et al*. (1996) and Salvo *et al*. (2008)
Aloe vera	matK, RbcL y ITS2	India, Germany, China, USA and Denmark	Treutlein *et al*. (2003) and Bold Systems (2015)
Sambucus mexicana	matK y ITS2	Belgium	Bold Systems (2015)
Agastache mexicana (Kunth) Lint and Epling	RbcL	Germany	Kaufmann and Wink (1994)
Valeriana officinalis	matK; RbcL y ITS2	USA, Spain, UK and Canada	Bell (2004) and Bold Systems (2015)
Smilax aspera L.	matK; RbcL y ITS2	Croatia, China, Spain, Portugal, UK and France	Li *et al*. (2011a, b), Schaefer *et al*. (2011) and Bold Systems (2015)

Perspective: The WHO recommends that traditional medicine, in which the herbalist plays a paramount role, should be evaluated and recognized as a genuine strategy for the treatment of the various diseases that occur in society. This implies conducting a series of studies in order to increase its efficiency, security, availability and that it be accessible to everyone (Article 10). To meet these objectives, the study of the genetic diversity of medicinal plants should be extended, in order to guarantee the identity of the plant materials being used. A simple way to accomplish this task is to use DNA barcoding markers. The DNA barcoding of medicinal plants can have other interesting applications. For instance, the authentication of medicinal plant material will help develop techniques for their propagation, thus counteracting the increasing loss of medicinal species from overexploitation in ecosystems. The propagation of medicinal plants will establish crops over large areas, which provide sufficient material for the production of high quality herbal formulas, increasingly demanded by the urban population.

The establishment of DNA barcode genetic diversity of Mexican medicinal plants may encourage the development of strategies to obtain varieties with increased amounts of active ingredients.

The medicinal plants sector in Mexico is still small but highly profitable for producers, collectors and industry (Juarez-Rosete *et al*., 2013). The implementation of a nationwide strategy to control and assure the quality of medicinal plants products through DNA barcoding markers, will detonate increased revenues from this sector.

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