Article

New *matK* initiators for life barcode in species of the genus Dasylirion

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Abstract

Dasylirion species are among the most important in the Chihuahuan Desert in ecological and economic terms. Its main use is the elaboration of a traditional alcoholic beverage called sotol. The genus comprises 22 species, not all suitable for this purpose and their identification is difficult. Molecular tools such as the barcode of life facilitate their identification and generate genetic information. There are universal primers for the *matK* gene, whose sequence is appropriate for life's barcode, which do not work for some species of this genus. The difference of a single nucleotide can cause problems in amplification. The objective of this work was to design and test new initiating sequences of the *matK* gene, which allow amplification in plants of the genus Dasylirion. The Primer3 Plus, FastPCR and the complete sequence of the *matK* gene of *D. wheeleri* were used for the design. The new oligonucleotides were tested with the species *D. texanum*, *D. leiophyllum*, *D. occidentalis* and *D. palaciosii*, with clean amplification of the *matK* gene (approximately 1 000 bp fragment) in each of them. These results contrasted with those obtained from the universal primers *matK* 390F and *matK* 1 326R, which generated secondary bands or failed amplification. Since the new initiators successfully amplified the *matK* gene in the studied species of Dasylirion, they are considered of value as tools for obtaining the barcode of life in sotol. Research conducted during the years 2016 to 2018.

Keywords: Dasylirion, arid zones, identification, oligonucleotides, sotol.

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Introduction

In Mexico, 40% of the national territory is made up of arid and semi-arid zones. They include a considerable part of the largest desert in North America, the Chihuahuan Desert, one of the regions with the highest species richness in the world (Granados-Sánchez, 2011). It houses around 6 000 plant species of varying sizes and complex shapes, with a high percentage (50%) of endemism (Rzedowsky, 1965; Sarukhan *et al*., 2009).

Since ancient times, desert species have been used for human consumption, as a building material, as natural barriers, as fiber, as fodder for livestock and recently in obtaining substances of economic interest such as gums, resins, latex, starch and secondary metabolites directed to medical applications (González-Medrano, 2012).

One of the genera with greater economic, ecological and cultural importance in arid areas is Dasylirion (Family Asparagaceae). These plants are commonly called sotoles and from them you get a traditional alcoholic beverage called 'sotol', which has protection of the designation of origin for Chihuahua, Coahuila and Durango (IMPI, 2002). However, not all species of this genus are suitable for that purpose.

Sotol plants are part of the rosetophile desert scrub (MDR), which represents approximately 12% of the total area of the Chihuahuan Desert (Rzedowski, 1965; Granados-Sánchez, 2011). They are characterized by having short and robust stems, long leaves screwed with spines on the edges. They have a floral escape of up to 5 m high and its fruit is an indehiscent capsule with a seed.

As an ecological component of the desert, it contributes to soil maintenance, while its leaves and seeds are food for birds and rodents (Reyes-Valdas *et al*., 2012). Its leaves are used in the elaboration of ornaments for patron celebrations and in times of drought as fodder for cattle, while its escape serves as construction material and in the manufacture of canes (Encina-Domínguez *et al*., 2013). There is evidence of its use as food by indigenous groups (Short *et al*., 2015), who cooked the stem in wells with hot stones and from the center already cooked obtained a flour to prepare rolls or cakes.

Currently 22 species of the genus Dasylirion are recognized distributed in arid and semi-arid mountainous areas of North America, from the southern United States of America to Oaxaca in Mexico (Bogler, 1998; The Plant List, 2013; Tropicos, 2018). The genus has been classified into different botanical families, including Liliaceae, Agavaceae, Nolinaceae, Ruscaceae and currently in the Asparagaceae family (Villaseñor, 2016; Tropicos, 2018).

Plants of this genus have distinctive features; one of them is the shape of the leaves, with variations in length and breadth, the orientation of the spines in the margin, the presence or absence of epicuticular wax, as well as the shape of its leaf surface (Bogler, 1994). Taxonomic recognition implies an adequate knowledge of the particular features between one species and another. The initiative to sequence a short DNA fragment that serves as a universal identifier, better known as the barcode of life, is an alternative in resolving the variation between Dasylirion species.

The search has focused on chloroplast genes (*matK*, *rbcL*, *rpo*C1 *trnH-psbA*, among others) but very few are effective individually, since they do not show enough variation (Jing *et al*., 2011). It was proposed to use the combination of *matK* and *rbcL* sequences as a barcode of life for angiosperms (CBOL, 2009). The above sequences have been used in the characterization of some species of the genus Dasylirion, including *D. serratifolium*, *D. wheeleri*, *D. miquihuanense* and recently *D. micropterum*, the latter recently described in the boundaries of the states of Coahuila and Nuevo León.

The *matK* and *rbcL* sequences for these species are recorded in the NCBI GenBank (Hebert *et al*., 2003; Reyes-Valdés *et al*., 2016; Villarreal-Quintanilla *et al*., 2016). The maturase K (*matK*) gene proposed by the Consortium for the Barcode of Life as an identification gene in conjunction with the *rbcL* gene, large subunit of ribulose-1,5-bisphosphate carboxylase, have given favorable results for a large number of plants (CBOL, 2009); however, the universality of these markers has been hampered by factors such as morphological, geographical variation, reticular evolution and lack of variation of these sequences in many plant species, which is why some researchers consider that the markers of life bars may not work for all genera of terrestrial plants (Roy *et al*., 2010), which leads, for example, to propose new highly variable regions within the *matK* gene (600 to 800 bp) with highly conserved alignment sites, avoiding the repetition of mononucleotides (Jing *et al*., 2011).

Some researchers have been given the task of designing specific initiators, within conserved regions of the *matK* gene, that are useful in identifying different taxonomic groups. Certain initiators have been designed specifically for Equisetum (Hausner *et al*., 2006), for the order of the Caryophylls (Cuenoud *et al*., 2002), among others. Jing *et al*. (2011) propose the initiators *matK* 472F and *matK* 1 248R as alternatives in the partial resolution of the problems presented when using the *matK* gene as a barcode of life. The initiators were successfully tested in 58 species from 47 families of angiosperm plants.

Some of these initiators that have been designed for certain taxonomic groups have been useful in identifying others. However, it is not ruled out that there are inconveniences.

The objective of this research was to design effective *matK* initiators in the identification of the species of the genus Dasylirion, whose amplification with universal initiators *matK* 390F (5'-CGA TCT ATT CAT TCA ATA TTT C - 3') and *matK* 1 326R (5' - TCT AGC ACA CGA AAG TCG AAG T – 3') (Cuenoud *et al*., 2002) generated bands of secondary type and poor quality sequences.

Materials and methods

Amplification with *matK* 390F and *matK* 1 326R initiators

The *matK* 390F and *matK* 1 326R initiators proposed by Cuenoud *et al*. (2002), were useful in the amplification of the *matK* gene in the species of *D. micropterum* KU535883.1 and *D. miquihuanense* KU535884.1, currently registered in the GenBank of the NCBI. However, they did not work for the species of *D. texanum*, *D. leiophyllum*, *D. occidentalis* and *D. palaciosii*. To determine the failure in the amplification process, modifications were made to the PCR technique.

First, aseptic conditions were ensured and PCR reagents were renewed. Subsequently, a 12 temperature gradient was performed taking as reference the alignment temperatures (Tm) proposed by the supplier (53.4 °C *matK* 390F and 60.8 °C *matK* 1326R). Under these conditions an amplification response was obtained at 52.2 °C. Subsequently, the DNA quality was verified, based on the absorbance ratio 260/280 with values between 1.6 to 1.8, the concentrations of the initiators were modified (right and left), the dNTP concentrations were increased, and the inclusion of Buffer with Mg and without Mg.

These modifications gave negative results or with some amplified intermittent bands for the species of *D. texanum*, D. *palaciosii* and *D. leyophyllum*. Only positive results were obtained in *D. occidentalis* and *D. micropterum*, the latter used as a control (+). However, at the time of sequencing, the amplification products were not suitable for processing.

Complementarity analysis with *matK* 390F and *matK* 1326R initiators

A complementarity analysis was performed using BLASTn (NCBI) for the *matK* 390F and *matK* 1 326R primers. The oligonucleotides were aligned with the complete sequences of the *matK* gene of *D. wheeleri* (HM640588.1) and *D. serratifolium* (HM640587.1) unique complete gene records for the genus Dasylirion in the GenBank (Kim and Kim, 2010). In particular, the existence of a match between the nucleotide bases of the primers and the sequences mentioned was tested.

***MatK* initiator design**

MatK primers were designed for the genus Dasylirion, with the help of Primer3Plus (Rozen and Skaletsky, 2000), FastPCR Professional 6.6 (Kalendar *et al*., 2017) and based on the complete sequence of the *matK* gene of the *D. wheeleri* species (HM640588.1) registered with Gen Bank (Kim and Kim, 2010). Of the generated initiators, those that presented the highest percentage of linguistic complexity and greater efficiency *in silico* PCR were evaluated in the FastPCR program.

When a nucleotide sequence is written as a text, it is possible to measure the repeatability of the letters. With this a measure known as linguistic complexity is obtained, under this criterion, the less repetitive DNA sequences will have greater complexity and vice versa. Additionally, it was sought that the amplified area coincided with most of the region interspersed between the two universal initiators (Jing *et al*., 2011).

Collection of plant material

Healthy and young leaves of at least six specimens were collected per species. Among them *D. texanum*, in Allende, Coahuila (28° 10’ 12.6” north latitude, 101°00’ 49.6” west longitude, 520 meters above sea level), *D. leiophyllum* in Sierra Mojada, Coahuila (27° 16’ 19.3” north latitude, 103° 38’ 2.9” west longitude, 1 344 meters above sea level), *D. occidentalis* in San Juan de Hornillos, Zacatecas (23° 03’ 645” north latitude, 103° 06’ 286” west longitude, 2 280 meters above sea level) and *D. Palaciosii* in Guadalcazar, San Luis Potosí (22° 37’ 57.8” north latitude, 100° 25’ 57.0” west longitude, 1 885 masl) (Figure 1).

Each specimen was labeled, georeferenced and the leaf samples were placed in paper bags for transport. Prior to DNA extraction, the material was washed with a 1% hypochlorite solution and subsequently with 70% ethanol for one minute. In the end, the excess was removed with sterile water and allowed to dry on paper towels.

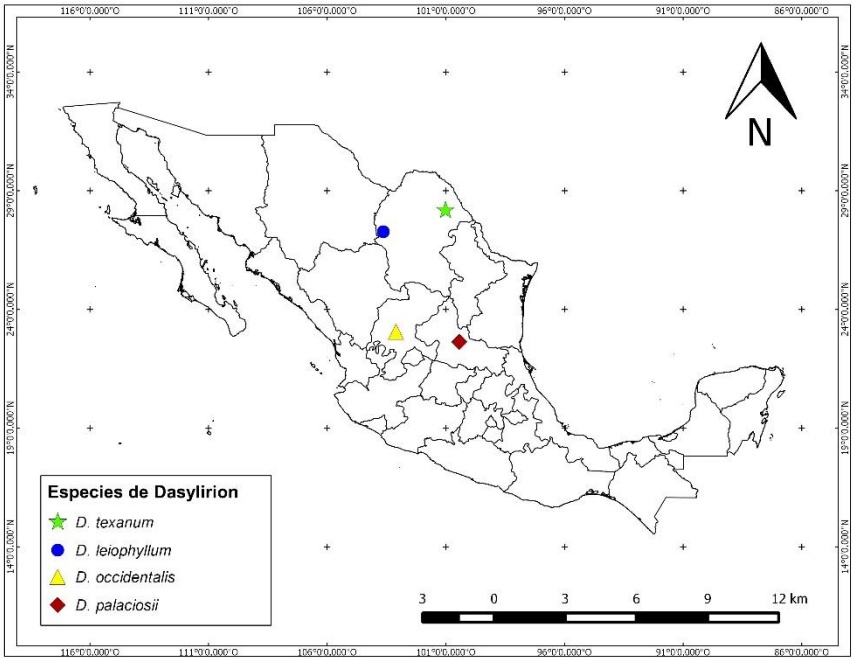

Figure 1. Collection sites of plant material of Dasylirion species. The localities include the states of Coahuila, Zacatecas and San Luis Potosí.

DNA extraction

To obtain the DNA of the different species, the methodology of Lopes *et al*. (1995) modified. 20 mg of fresh tissue was placed in a sterile mortar and ground with liquid N_2, the resulting powder was placed in a 2 mL eppendorf tube and 800 µL of lysis buffer (100 mM Tris-HCl at pH= 8, was added, 20 mM NaCl, 20 mM EDTA pH= 8 and 1% N-Lauryl-sarcosine), mixed thoroughly and allowed to stand for 10 min. 800 µL of phenol (Sigma, Phenol Solution, equilibrated with 10 mM Tris HCL, pH 8, 1 mM EDTA) was added for molecular biology, stirred in a vortex and centrifuged for 20 min at 4 °C at 12 000 rpm.

The aqueous phase was recovered in a new eppendorf tube and 100 µL of RNAse in 10 mg mL^{-1} concentration was added and mixed by inversion. 800 µL of cold isopropanol (-20 °C) was added, mixed to form the skein of DNA, which was collected with a sterile Pasteur pipette hook and transferred to a new 1.5 mL eppendorf tube. The tablet was washed with 200 µL of 70% alcohol (-20 °C) and decanted. It was allowed to dry for 15 min and was resuspended in sterile distilled water, where it was kept refrigerated at -20 °C. For visualization, a 1% agarose gel was run, with the addition of the GelRed Nucleid Acid Biotum dye, in a proportion of 1 µL per 10 mL of gel. The DNA samples obtained were quantified by means of NanoDrop ND-1000 (Software ND-1000) and from the reading's DNA dilutions were prepared at 50 ng μL^{-1} in sterile water.

Amplification with new initiators

To test the efficiency of the new initiators, the amplification process was performed for each Dasylirion species. The polymerase chain reaction (PCR) was carried out in a volume of 50 µL. Each reaction contained 5 µL of 10X *Taq* Standard Buffer, 1 µL of 10 mM dNTPs, 1 µL of 10 µM Forward initiator, 1 µL of 10 µM Reverse initiator, 0.25 µL of *Taq* polymerase, 37.75 µL of nuclease-free water and 4 µL of tempered DNA (dilution 50 ng μL^{-1} of DNA in sterile water), according to the BioLabs kit protocol (*Taq* DNA Polymerase with Standard *Taq* Buffer, the latter with Mg included).

To obtain an optimum alignment temperature (Tm) for the new initiators, a gradient PCR of 12 different temperatures was run, including those recommended by the supplier (56.7 °C *matK* 335F and 57. 7 °C *matK* 1327R). The best response was obtained with the temperature of 57.8 °C. From this result, an end-point PCR of 40 cycles was performed, with three different temperatures: initial denaturation phase 94 °C for 4 min, denaturation phase 94 °C for 30 s, hybridization phase 57.8 °C for 35 s, extension phase 72 °C for 35 s and final extension phase 72 °C for 10 min.

This procedure was carried out in an Axigen Maxigene thermal cycler. To visualize the PCR products, a 1% agarose gel electrophoresis was run (for each species), with the use of the 0.5 X TBE buffer, added with the GelRed Nucleid Acid Biotum dye in proportion of 1 µL per 10 mL gel. To load the samples, 5 µL of PCR product was mixed with 3 µL of loading buffer (TrackIt™ Cyan/Orange Loading Buffer). 1 Kb Plus DNA Ladder - Life Technologies was used as molecular weight marker. The electrophoresis was run in a horizontal chamber at 115 Volts for 30 min. The gel was observed in Enduro TM GDS photodocumenter of the brand Labnet International, Inc.

Sequencing

As further evidence of the quality of the amplicons obtained and to analyze whether they work for the barcode approach, the PCR products of *D. texanum* were sequenced in the Genomic Services Laboratory (LabSerGen) of the Advanced Genomics Unit -CINVESTAV, Irapuato, Guanajuatoto. A 3 730xl DNA Analyzer (Applied Biosystems) sequencer was used and sequencing was done both ways.

Nucleotide sequence quality

The sequences obtained from the amplification with the new primers (*matK* 335F and *matK* 1 327R) were evaluated, through their electropherograms with the Finch TV program version 1.4.0. The program provides a quality value called 'quality value Q'. This value calculated as the base 10 logarithm of the probability of error multiplied by -10, represents the feasibility of identifying a nitrogen base at a specific position within a sequence.

In this way values of Q= 10 (Q10), represent the probability of error of one in ten (1/10), Q= 20 (Q20), the probability of error of one in one hundred (1/100), Q= 30 (Q30), the probability of error of one in thousand (1/1000) and Q= 40 (Q40) the probability of error of one in 10 000 (1/10 000). Sequences with average values of Q= 30 or higher were considered of good quality (NABR, 2012).

Results and discussion

Evaluation of the *matK* 390F and *matK* 1 326R initiators

Obtaining a successful amplification or PCR process depends on a considerable number of variables, among which are the conditions of the apparatus where the reaction is carried out and the functionality of the reagents involved in the reaction itself. If the process fails, certain modifications to the technique can be made, which help to make the process more efficient (Espinosa-Asuar, 2007). Among the variables that were reviewed to test the amplification of the *matK* 390F and *matK* 1 326R primers is the quality of the DNA used, with absorbance ratio values 260/280= 1.6 to 1.8, indicative of good quality (low amount of protein).

PCR reagents were renewed (dNTPs, *Taq* polymerase, ultra-pure water, buffer with and without Mg), discarding possible sources of contamination and variants were tested in the concentrations of each reagent. The material used as tubes, tips and containers, were autoclaved, the laminar flow hood was disinfected, and the products obtained were visualized on agarose gel.

In Figure 2 four electrophoretic patterns are presented. Each shows the PCR products from the *matK* 390F and *matK* 1 326R initiators (Cuenoud *et al*., 2002) for each Dasylirion species. Figures 2a and 2b correspond to the species *D. palaciosii* and *D. texanum*, respectively. Both images show the absence of amplified bands, except in the case of positive controls (C+) corresponding to *D. micropterum*, whose sequence recorded in the GenBank (Reyes-Valdes *et al*., 2016, KU535883.1) was obtained, from the initiators *matK* 390F and *matK* 1 326R, where the presence of amplicon is evident.

Figures 2c and 2d correspond to the species *D. occidentalis* and *D. leiophyllum*. Bands with greater definition are appreciated, especially in the *D. occidentalis* species, where samples of seven specimens were amplified. However, in *D. leiophyllum* the banding is intermittent and shows weak bands marked alternately.

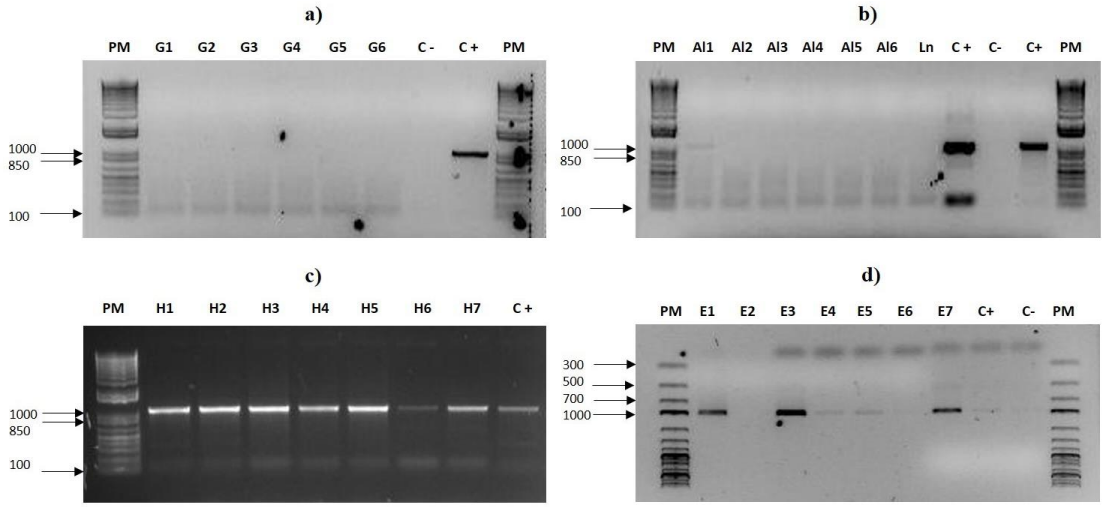

Figure 2. PCR results of a partial sequence of the *matK* gene from the pair of primers *matK* 390F and *matK* 1 326R (Cuenoud *et al*., 2002) in species of the genus Dasylirion. a) *D. palaciosii*; b) *D. texanum*; c) *D. occidentalis*; and d) *D. leiophyllum*.

The bands obtained in the different gels (Figure 2), corresponding to *D. micropterum* as a positive control (C +), are evidence that the initiators *matK* 390F and *matK* 1 326R are not degraded and are effective for that species. While the presence of intermittent bands, together with obtaining poor quality sequences with average values of Q < 30, are proof that the initiators are not effective in the remaining species.

In order to carry out an effective PCR process, it is necessary that one of the initiators has the same sequence that is in one of the DNA chains and the other initiator has the complementary sequence that will be at the end of the fragment to be amplified, If this is not the case, the selected site cannot be effectively amplified (Espinosa-Asuar, 2007).

The *matK* gene is one of the fastest evolving coding sequences of the plastid genome. However, it has low amplification and sequencing rates due to the low universality of the primers (Hilu and Liang, 1997; Hollingsworth *et al*., 2011; Jing *et al*., 2011). This characteristic may be the main cause of the absence of bands, weak banding and the presence of secondary bands (Figure 2b and Figure 2d) in the amplification with the universal primers of the different Dasylirion species.

Complementarity analysis

In Figure 3 the results of analysis of complementarity between the oligonucleotide and *matK* 390F sequences *D. wheeleri* and *D. serratifolium* through alignment with BLASTn presented. It was found that there is a difference of two nitrogen bases by comparison. The *matK* 390F initiator when aligning with the complete *Dasyliron* sequences differs with position 405 corresponding to an Adenine (A) and with position 417 corresponding to a Thymine (T). The reverse initiator (*matK* 1 326R) presented complete complementarity. To the lack of alignment between nitrogen bases of the *matK* 390F initiator, the failure in the process of amplification of the *matK* gene in Dasylirion species is attributed.

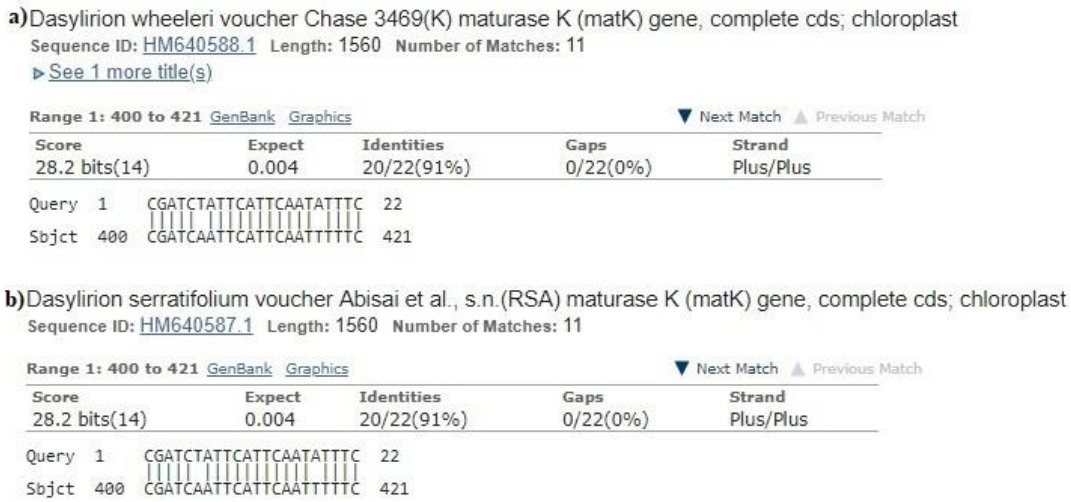

Figure 3. a) Alignment of *matK* 390F initiator with respect to the complete *matK* gene sequence of the *D. wheeleri* species registered in GenBank-NCBI; and b) Alignment of the *matK* 390F initiator with respect to the complete *matK* gene sequence of the *D. serratifolium* species registered in the GenBank.

New *matK* starters

In Table 1, the two pairs of primers designed for amplification of the *matK* gene in Dasylirion species are shown. The pairs with high values of linguistic complexity and efficiency *in silico* PCR were chosen (Kalendar *et al*., 2017). Both pairs were synthesized by Eurofins Genomics in concentrations of 100 µM.

Table 1. *MatK* initiators generated and tested in FastPCR.

Initiator	Sequence (5'-3')	Linguistic complexity	PCR efficiency *in silico*
		(%)	
matK 335F	tttcattctcgttgcgattag	85	80
matK 1327R	actttattcgatacaaaccctgtt	93	80
matK 413F	caatttttccctttttagaggaca	79	81
matK 1409R	gaacccaatcttcgcaaaa	71	79

Amplification with new *matK* initiators

The proposed initiator pairs (Table 1) were tested in the amplification of *matK* fragments for Dasylirion species. The pair with less linguistic complexity, *matK* 413F and *matK* 1 409R, did not produce amplification bands for Dasylirion species. The *matK* 335F and *matK* 1 327R pair gave satisfactory results in the amplification of the *matK* gene in the four species, with well-defined bands at the approximate position of 1 000 bp (1 Kb PM Marker) and without the presence of secondary bands (Figure 4).

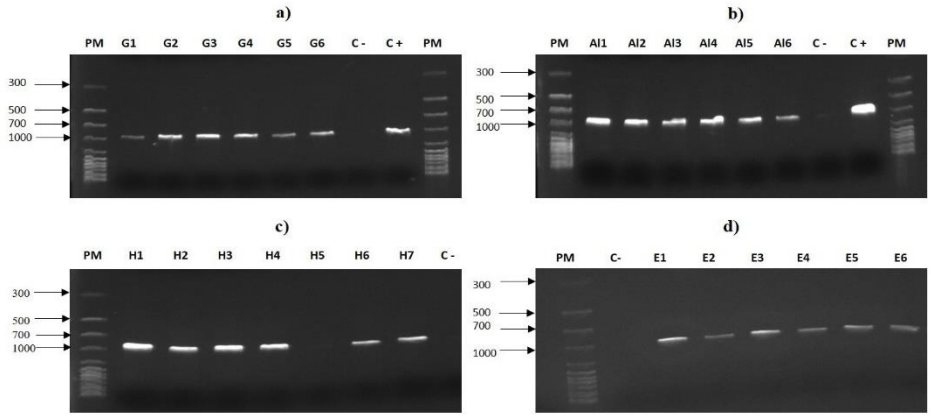

Figure 4. PCR results of a partial sequence of the *matK* gene from the pair of designed primers *matK* 335F and *matK* 1 327R in species of the genus Dasylirion. a) *D. palaciosii*; b) *D. texanum*; c) *D. occidentalis*; and d) *D. leiophyllum*.

Sequencing

The sequences obtained from *D. texanum* amplicons showed to be of good quality. The average quality values (Q) fluctuated between 36 and 64, which is why they were considered suitable for life bar codes (Jing *et al*., 2011; NABR, 2012). The sequences were uniform with each other.

Alignment of the Al-01 sequence of *D. texanum* with *matK* sequences of the Dasylirion group (taxid: 39504, GenBank-NCBI), showed that the four recorded species can be discriminated (Table 2).

Table 2. Nucleotide substitutions between the Al-01 sequence (*D. texanum*) with respect to the *matK* sequences of Dasylirion species registered in GenBank.

Species	GenBank registration	Number of substitutions
D. miquihuanense	KU535884.1	1
D. serratifolium	HM640587.1	1
D. serratifolium	AB029800.1	1
D. wheeleri	HM640588.1	2
D. micropterum	KU535883.1	3

The sequence of *D. texanum* (Al-01) presented 99% identity with respect to the sequences of the Dasylirion group, with differences of 1 to 3 nucleotide substitutions between species by comparison: a substitution with respect to *D. miquihuanense* (Reyes-Valdés *et al*., 2016) and *D. serratifolium* (Tamura, 1999; Kim and Kim, 2010), two substitutions with respect to *D. wheeleri* (Kim and Kim, 2010) and three substitutions with *D. micropterum* (Reyes-Valdés *et al*., 2016). The high percentage of identity between the problem sequence and the GenBank reference sequence, as well as the number of nucleotide substitutions found by alignment, illustrate the value of the *matK* 335F and *matK* 1 327R primers, for life barcode with *matK* in Dasylirion.

Conclusions

The difference of two nitrogenous bases in one of the universal primers (*matK* 390F) with respect to the sequence of the complete *matK* gene of *D. wheeleri* (GenBank), is the attributable cause in the failure of the amplification process of the *matK* gene in the species of Dasylirion under study. The *matK* 335F and *matK* 1 327R primers designed in this investigation, allowed the amplification of fragments close to 1 000 bp in all Dasylirion studied species (*D. occidentalis*, *D. palaciosii*, *D. texanum*, *D. leiophyllum*).

The sequences obtained, of each Dasylirion species, presented average values of Q between 36 and 64, which are considered fragments of sufficient quality to obtain a barcode of life. The sequences of *D. texanum* were uniform among themselves and with variation in nucleotide substitution with respect to the *matK* sequences (genus Dasylirion) recorded in the GenBank. The results indicate that the new oligonucleotides (*matK* 335F and *matK* 1 327R) are of value for obtaining a barcode of life in species of the genus Dasylirion. In particular, the four species referenced in the GenBank and *D. texanum*, studied in this work, can be differentiated from each other with the partial sequence of the *matK* gene.

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