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Evaluation of plastid and nuclear DNA markers in barcoding of *Aloe saudiarabica*, KSA

Awad A Algarni*

Department of Biology, College of Science and Arts, Al-Baha University, Baljurashi, Saudi Arabia

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ABSTRACT

There is great plant diversity in Saudi Arabia. The Asphodelaceae family is within this great diversity, especially the rare species such as the plant, *Aloe saudiarabica*. Such plants must be preserved in their natural ranges, hence, the need to document them. Genetic markers have become the approved and widely used method for documenting rare plants. The current study deals with the use of three genetic markers to document *A. saudiarabica* for the first time. The used genetic markers were Maturase-K (*matK*), Ribulose-bisphosphate-carboxylase (*rbcL*), and Internal-transcribed-spacer (ITS). The study found that the primers used for the *rbcL* gene were not effective in achieving identification. Sequencing of the *matK* and ITS were achieved successfully. The sequences were determined for both markers using two pairs of primers and deposited in the NCBI databases (GenBank). These markers were effective in identifying *A. saudiarabica* and determining its evolutionary relationship with other *Aloe* species in various databases. The study showed that *A. vera* is high similar (>99%) to the other species. In conclusion, the study showed the likelihood of the different genetic markers to document *A. saudiarabica*, especially the currently investigated *matK* and ITS.

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Introduction

Saudi arabia boasts a rich and diverse flora, which harbors valuable yet largely undiscovered plant resources. Therefore, there is a pressing need to conserve these plant resources to ensure their long-term sustainability and continued use in various fields, such as medicine, agriculture, and industry (1).

The *Aloe* genus is a group of succulent plants that belongs to the Asphodelaceae family. There are over 500 species of *Aloe* plants, and they are distributed in various regions of the world, including Saudi Arabia (2).

A number of rare and endemic species, including *A. saudiarabica*, are found in this genus. Therefore, documenting the genetic diversity of these plants and conducting phenotypic studies are of utmost importance to accurately describe and differentiate between species, understand the evolutionary history and ecological significance of the *Aloe* genus, and develop conservation strategies to ensure the preservation of these valuable plant resources for future generations (3, 4).

The *A. saudiarabica* is an indigenous plant of Saudi Arabia, with distinguished appearance characteristics that have been documented in references (5,6). In this study, we report the first genetic documentation of this plant. Accurate and detailed inventories of different plant species are crucial for protecting and preserving the diversity of plant life. However, traditional methods of identifying plants based on physical characteristics can be challenging. Therefore, it has become essential to utilize new technological and analytical methods in order to better understand and safeguard plant biodiversity (7-9).

DNA barcoding is an efficient method for identifying medicinal plant species, which can play a crucial role in both the conservation and use of these plants. Compared to traditional morphological identification methods, DNA barcoding allows for more accurate identification of species, which ensures that the products used for medicinal purposes are genuine. This is particularly valuable for medicinal plant species (10,11). Conserved DNA regions have been identified in different plant species through DNA analysis, which has resulted in the discovery of numerous genetic markers since the development of plant DNA barcoding (12).

Among the highly adopted markers in previously conducted studies include the genes of the plastids and the nuclear ITS region. Also, the combination of the two approaches was considerable for proper species allocation (13,14). The present study has focused on the use of genetic markers, specifically *rbcL*, *matK*, and ITS, for the purpose of identifying *A. saudiarabica*. The efficacy of identification through the aforementioned markers was evaluated in this study.

Materials and Methods

Samples locality

Specimens of plants were gathered from the location of Baljurash, Heznah; Albaha region in Saudi Arabia (19°50'17.3"N 41°32'55.7" E) in 2020. The characteristic morphology of the plant leaves was the basis to distinguish and confirm its identity. The collected plant leaves shown in (Figure 1) were used to carry out this investigation.

Molecular Identification

DNA Extraction

The NucleoSpin Plant II kit was utilized to extract

* Corresponding author. Email: awad-algarni-j@hotmail.com

Figure 1. *Aloe saudiarabica* leaves.

DNA from the plant leaves, following the protocols outlined in the kit's manual. The integrity of the purified DNA was confirmed by gel electrophoresis using Bio-Rad.

Amplification

Table (1) lists all the primers employed in the PCR reaction. The PCR reaction was carried out using Thermo Scientific master mix and all guidelines for mixing and cycling were strictly adhered to. The PCR amplification was carried out using a GenAmp 9700 thermal cycler from Applied Biosystems. Following PCR amplification, the products were purified using a protocol from GE-Healthcare, specifically ExoSAP-IT. The PCR product purity was subsequently confirmed by electrophoresis on a 1.2% agarose gel, using a 100 bp Bioatlas DNA marker as the molecular standard. The gel was visualized using ultraviolet light and imaged using a gel documentation system from Bio-Rad.

Sequencing

The sequencing reaction was performed using the Applied Biosystems BigDye Terminator protocol, using the same primers listed in Table 1. The sequencing products were then analyzed using an ABI3500 genetic analyzer from Applied Biosystems.

Analysis of data

The obtained forward and reverse sequences were assembled and then used in identification and analysis. These sequences were submitted to the NCBI (GenBank) databases for deposition. The identification and evolutionary analysis were conducted by two methods for confirmation. The used databases were the BOLD and NCBI systems. The MEGA-X software was finally used in creating the evolutionary trees (15).

Results

In this study, genetic detection was performed using the primers listed in Table (1). The approximate molecular sizes of all fragments were as predicted except the *rbcL* gene. No fragment was generated in the PCR of the *rbcL* gene. The rest markers gave positive results, as arranged in Table (1). The molecular sizes of *matK* fragment by the 1st primer pair was 900 bp, while an 850 bp fragment was generated by the second pair. Likewise, the ITS (1) and (2) primers gave weights of 800 and 500, respectively.

Partial sequencing of *matK* and ITS has been achieved by the same PCR primers. All the obtained sequences were then assembled and deposited in GenBank. *A. saudiarabica* identification through the GenBank and the BOLD approaches was accomplished. The neighboring species retrieved from the GenBank were used to construct the phylogeny relationships. *A. saudiarabica* was documented genetically for the first in this study.

Based on the identification systems and the aforementioned sequences, *Aloe* was identified as the dominant genus, with *A. vera* being the most closely related species, followed by various other *Aloe* spp. The evolutionary trees were constructed using MEGA-X software with 1000 replicates for the Bootstrap values, and nucleotide sequence

Table 1. List of Primers used for PCR and sequencing.

Barcode region	Primers		Sequence (5′-3′)	Reference
rbcL	1 F		ATGTCACCACAAACAGAAAC	(28)
	724 R		TCGCATGTACCTGCAGTAGC	
matK	XF	1th	TAATTTACGATCAATTCATTC	(29)
	MALP-R1		ACAAGAAAGTCGAAGTAT	
	1Rkim F	2th	ACCCAGTCCATCTGGAAATCTTGGTTC	
	3Fkim R		CGTACAGTACTTTTGTGTTTACGAG	
ITS 1	5a F		CCTTATCATTTAGAGGAAGGAG	(30)
	4 R		TCCTCCGCTTATTGATATGC	
ITS 2	S2F		ATGCGATACTTGGTGTGAAT	(31)
	S3R		GACGCTTCTCCAGACTACAAT	

Table 2. The NCBI blast result for the *Aloe saudiarabica matK* sequences (first primer).

No.	Species name	sequence (bp)	Query cover	E-value	Indent.	Accession
1	*Aloe saudiarabica*	835	100%	0	100%	MZ488573
2	*Aloe vera*	817	97%	0	99.88%	KX377524
3	*Aloe vera*	817	97%	0	99.88%	KY556640
4	*Aloe vera*	817	97%	0	99.88%	JQ276402
5	*Aloe vera*	811	97%	0	99.88%	MW176075

alignments were performed using ClustalW.

The 1st primer pairs of the *matK* gene, *matK*-XF and *matK*-MALP-R1, gave a sequence of 835 bp in length. *A. vera* was the first species in the BOLD identification system with 99.88 % similarity. The same species with the same percentage was also obtained using the GenBank tools (Table 2). Figure (2) shows the phylogenetic tree constructed, including the neighboring species, for this sequence with a total mean distance of 0.1 (standard error, 0.0). Table (3) reveals the accompanied substitution matrix with this tree. Aligned sequences of *A. saudiarabica* and the closest relative *A. vera* are shown in Figure (3).

The 2nd primer pairs of the *matK* gene, 1R_ kim and 3F_ kim, gave a sequence of 824 bp in length. *A. vera* was the first species in the BOLD identification system with 99.88% similarity. The same species with the same percentage was also obtained using the GenBank tools (Table 4). Figure (4) shows the phylogenetic tree constructed including the neighboring species for this sequence with a total mean distance of 0.1 (standard error, 0.0). Table (5) reveals the accompanied substitution matrix with this tree. Aligned sequences of *A. saudiarabica* and the nearest relative *A. vera* is shown in Figure (5).

Concerning sequencing of ITS, the ITS1 pair of primers gave 395 bp sequence. *A. dorotheae* came first through BOLD identification with 98.63% similarity.

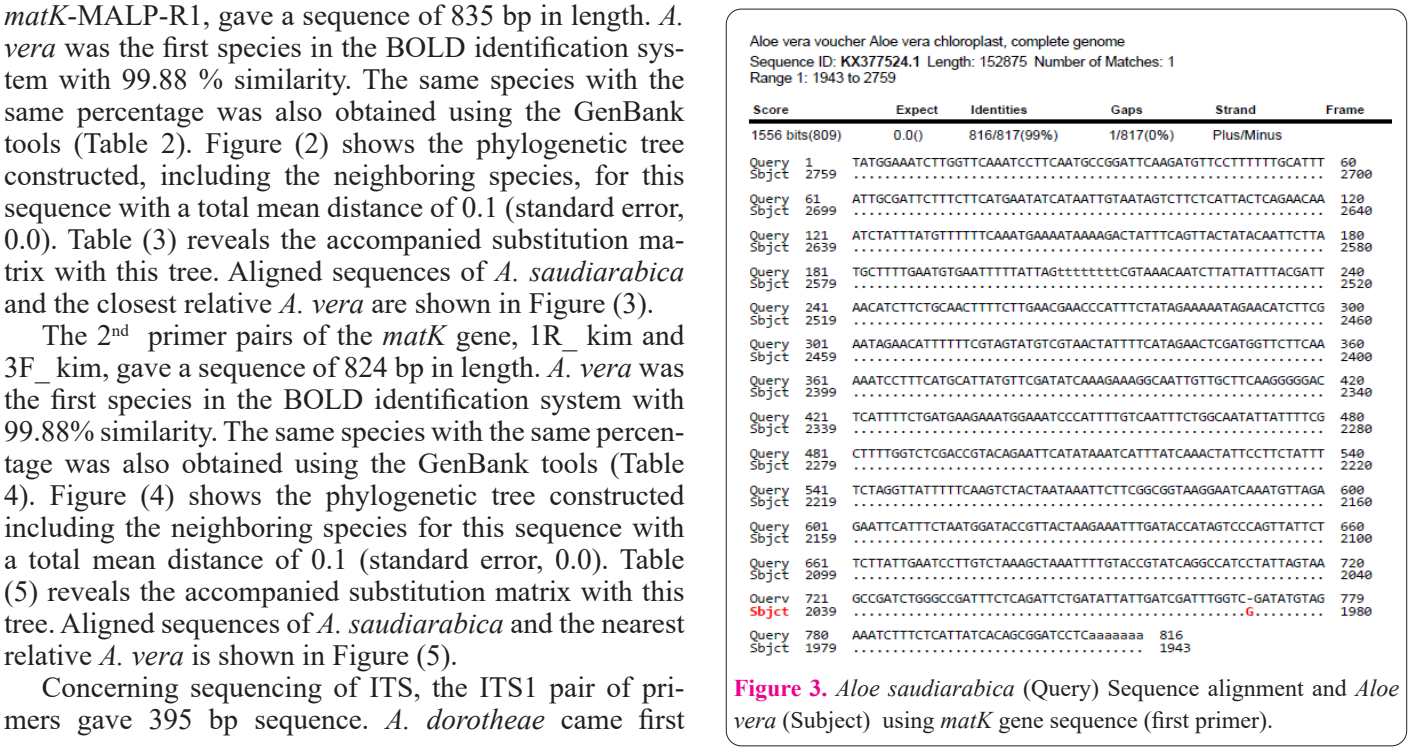


Figure 3. *Aloe saudiarabica* (Query) Sequence alignment and *Aloe vera* (Subject) using *matK* gene sequence (first primer).

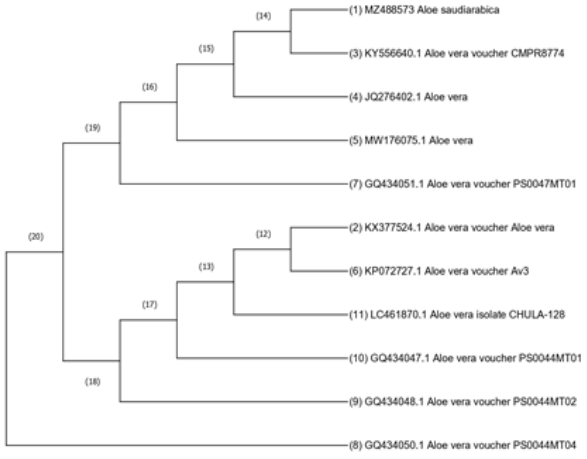


Figure 2. Phylogenetic tree of *Aloe saudiarabica* using matK sequences (first primer).

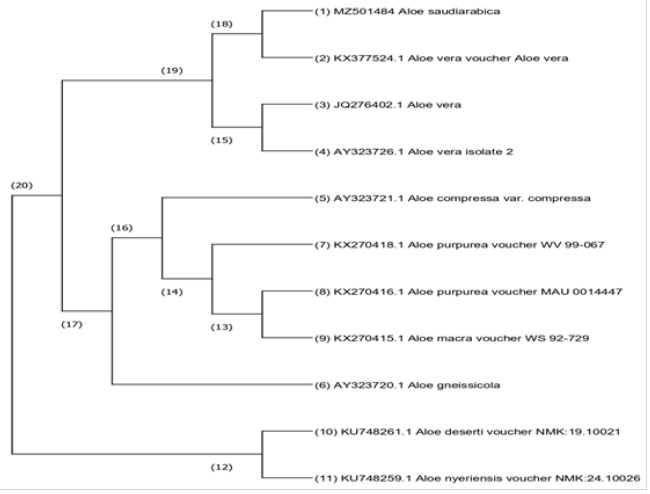


Figure 4. Phylogenetic tree of *Aloe saudiarabica* using matK sequences (second primer).

Table 3. substitution estimates for matK sequence (first primer).

From\To	A	T	C	G
A	-	7.4830	3.2404	9.4076
T	5.9411	-	9.5760	2.7976
C	5.9411	22.1141	-	2.7976
G	19.9782	7.4830	3.2404	-

Table 5. substitution estimates for matK sequence (second primer).

From\To	A	T	C	G
A	-	8.3729	3.6465	9.2094
T	6.7410	-	8.3080	3.1792
C	6.7410	19.0763	-	3.1792
G	19.5271	8.3729	3.6465	-

Table 4. The NCBI blast result for the *Aloe saudiarabica matK* sequences (second primer).

No.	Species name	sequence (bp)	Query cover	E-value	Indent.	Accession
1	*Aloe saudiarabica*	824	100%	0	100%	MZ501484
2	*Aloe vera*	824	100%	0	99.88%	KX377524
3	*Aloe vera*	824	100%	0	99.88%	JQ276402
4	*Aloe vera*	823	100%	0	99.76%	AY323726
5	*Aloe compressa* var. *compressa*	823	100%	0	99.51%	AY323721

Table 6. The NCBI blast result for the *Aloe saudiarabica* ITS1 sequences.

No.	Species name	sequence (bp)	Query cover	E-value	Indent.	Accession
1	*Aloe saudiarabica*	395	100%	0	100%	MZ474878
2	*Aloe retrospiciens*	484	92%	0	98.63%	KJ557911
3	*Aloe dorotheae*	364	92%	0	98.63%	KJ557867
4	*Aloe camperi*	364	92%	0	98.63%	KJ557857
5	*Aloe sinkatana*	364	92%	0	98.63%	KC893738

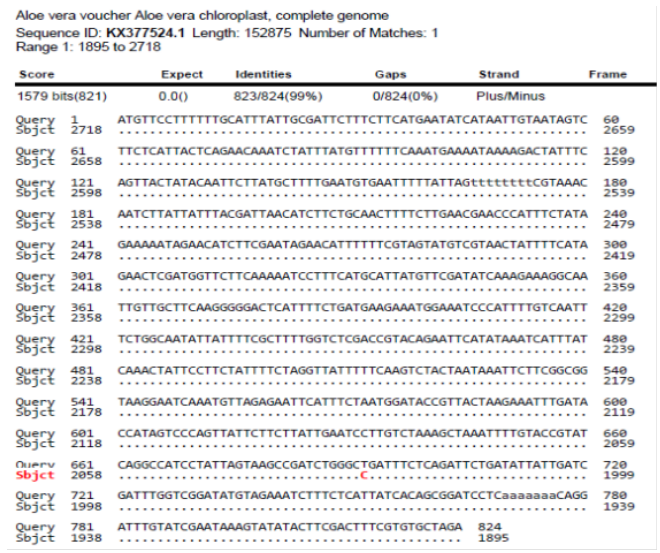


Figure 5. *Aloe saudiarabica (Query) Sequence alignment and Aloe vera (Subject) using matK gene sequence* (second primers).

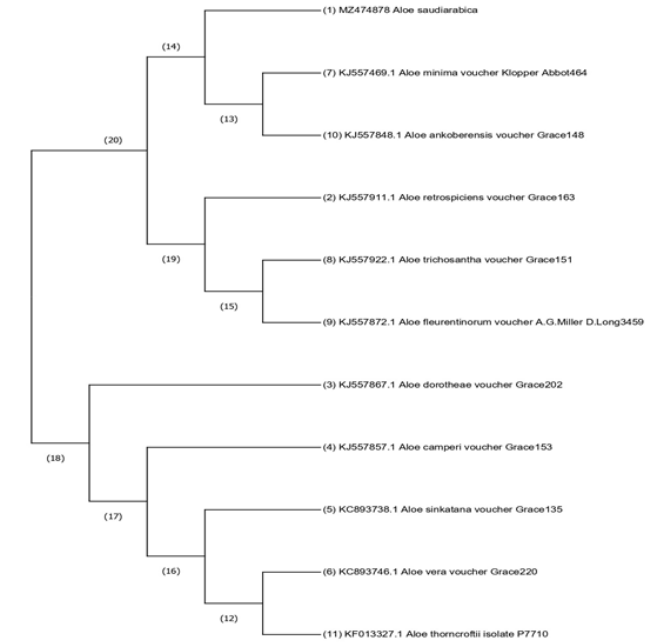


Figure 6. Phylogenetic tree of *Aloe saudiarabica* using ITS1 sequences.

Meanwhile, *A. retrospiciens* shown in table (6) was the first matched species in the gene bank by 98.63%. Figure (6) shows the tree of relatedness for this sequence with neighboring species. Also, table (7) represents substitution estimates for this sequence. A total tree distance of 0.01 was recorded between *A. saudiarabica* and its relatives. Aligned sequences of *A. saudiarabica* and the closest relative *A. retrospiciens* is shown in figure (7).

On the other side, the ITS2 primers (ITS S2F & ITS S3R) gave 375 bp sequence. Identification by BOLD produced *Kumara haemanthifolia* (formerly called *A. haemanthifolia*) as the nearest species, with a score and similarity percentage of 328 bp and 95.37%, respectively. *A. dorotheae* came in the third rank with a score and similarity percentage of 321 bp and 99.69%, respectively. *A. vera* shown in table (8) was the nearest species in the gene bank identification system with 99.73%. Figure (8)

Table 7. substitution estimates for ITS sequence.

From\To	A	T	C	G
A	-	4.4566	8.7423	8.5029
T	4.7458	-	21.8625	9.0775
C	4.7458	11.1449	-	9.0775
G	4.4454	4.4566	8.7423	-

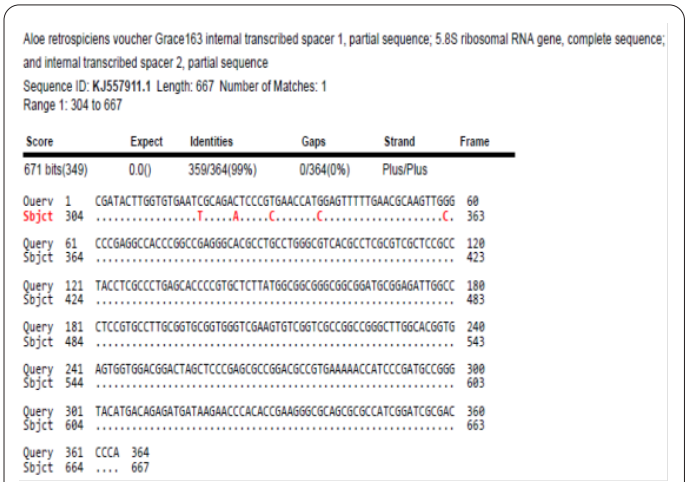


Figure 7. *Aloe saudiarabica* (Query) Sequence alignment and *Aloe vera* (Subject) using ITS1 sequence.

Table 8. The NCBI blast result for the *Aloe saudiarabica* ITS2 sequences.

No.	Species name	sequence (bp)	Query cover	E-value	Indent.	Accession
1	*Aloe saudiarabica*	375	100%	0	100%	MZ474879
2	*Aloe vera*	366	97%	0	99.73%	MN519271
3	*Aloe vera*	365	97%	0	99.45%	MK087867
4	*Aloe nyeriensis*	366	97%	0	98.91%	MT137508
5	*Aloe kedongensis*	366	97%	0	98.63%	MT137515

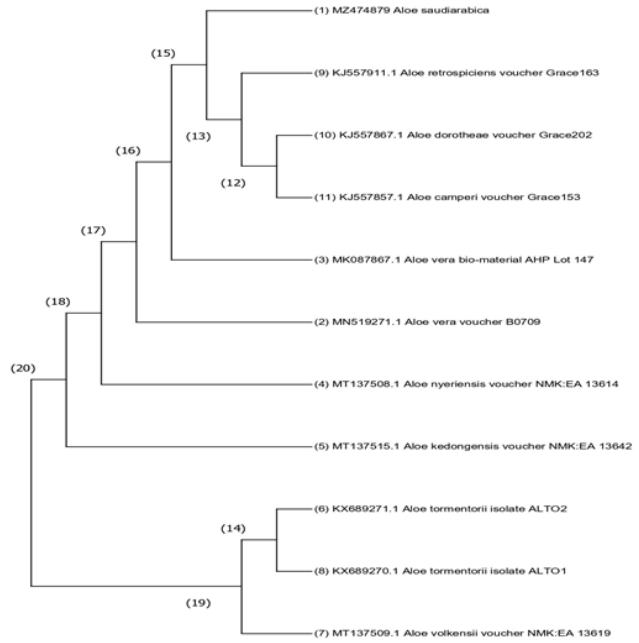


Figure 8. Phylogenetic tree of *Aloe saudiarabica* using ITS2 sequences.

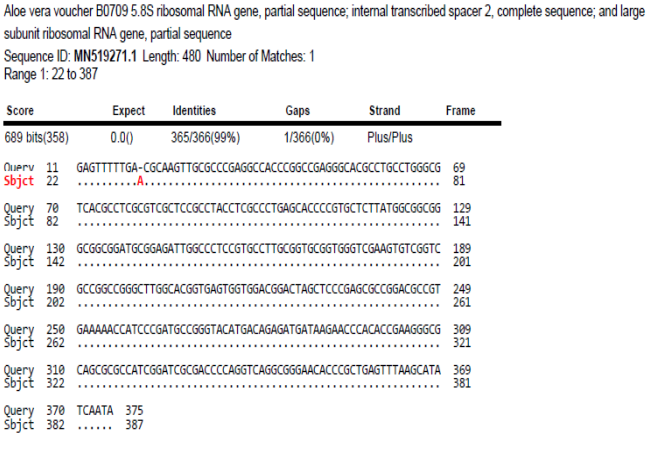
Table 9. substitution estimates for ITS2 sequence.

From\To	A	T	C	G
A	-	2.7596	5.8555	6.3442
T	3.1281	-	37.0835	6.2056
C	3.1281	17.4768	-	6.2056
G	3.1980	2.7596	5.8555	-

shows the tree of relatedness for this sequence with neighboring species. Also, table (9) represents substitution estimates for this sequence. A total tree distance of 0.01 was recorded between *A. saudiarabica* and its relatives. Aligned sequences of *A. saudiarabica* and the closest relative *A. vera* is shown in Figure (9).

Table 10 presents a compilation of the most closely related species to *A. saudiarabica*, as determined by the two techniques of identification applied. Each matK sequence (obtained from each pair of primers) was aligned and combined into a single piece of approximately 784 base pairs in length, as shown in Figure 10. The same was done concerning the ITS sequences which also aligned into one piece of about 335 bp (Figure 11). With the utilization of both identification methods and by comparing the last matK sequence, *A. vera* was found to be the nearest relative to *A. saudiarabica*, displaying a high degree of similarity at over 99%. Furthermore, ITS revealed a high similarity of greater than 99% between *A. saudiarabica* and *A. vera* in GenBank.

Using BOLD platform, *A. dorotheae* was identified as the most similar species to *A. saudiarabica* with a similarity of over 99%, followed by various other species of *Aloe*. With an equivalent level of similarity, *A. vera* was ranked as the 24th most similar species. In all these species, the highest length of the sequence recorded was 321 bp for the first species and 309 bp for *A. vera*.


Aloe vera voucher B0709 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: **MN519271.1** Length: 480 Number of Matches: 1
Range 1: 22 to 387

Score	Expect	Identities	Gaps	Strand	Frame
689 bits(358)	0.0()	365/366(99%)	1/366(0%)	Plus/Plus	

```
Query  11   GAGTTTTTGA-CGCAAGTTGCGCCCGAGGCCACCCGGCCGAGGGCACGCCTGCCTGGGCG  69
Sbjct  22   ..........A.............................................................  81

Query  70   TCACGCCTCGCGTCGCTCCGCCTACCTCGCCCTGAGCACCCCGTGCTCTTATGGCGGCGG  129
Sbjct  82   ..........A.............................................................  141

Query  130  GCGGCGGATGCGGAGATTGGCCCTCCGTGCCTTGCGGTGCGGTGGGTCGAAGTGTCGGTC  189
Sbjct  142  ..........A.............................................................  201

Query  190  GCCGGCCGGGCTTGGCACGGTGAGTGGTGGACGGACTAGCTCCCGAGCGCCGGACGCCGT  249
Sbjct  202  ..........A.............................................................  261

Query  250  GAAAAACCATCCCGATGCCGGGTACATGACAGAGATGATAAGAACCCACACCGAAGGGCG  309
Sbjct  262  ..........A.............................................................  321

Query  310  CAGCGCGCCATCGGATCGCGACCCCAGGTCAGGCGGGAACACCCGCTGAGTTTAAGCATA  369
Sbjct  322  ..........A.............................................................  381

Query  370  TCAATA  375
Sbjct  382  ......  387
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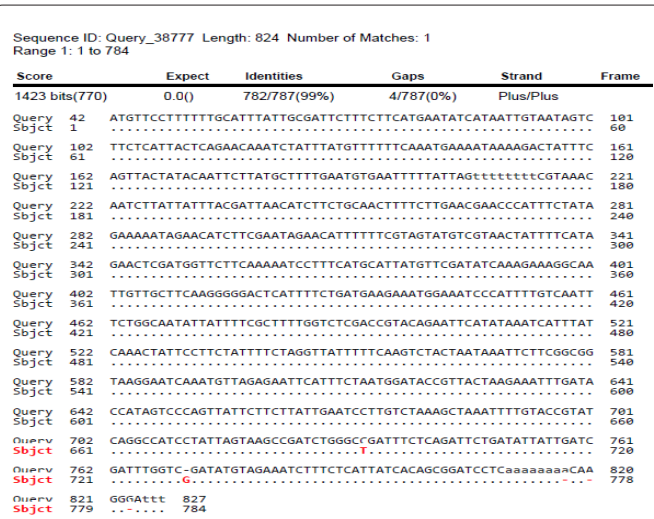
Figure 9. *Aloe saudiarabica* (Query) Sequence alignment and *Aloe vera* (Subject) using ITS2 sequence.


Sequence ID: Query_38777 Length: 824 Number of Matches: 1
Range 1: 1 to 784

Score	Expect	Identities	Gaps	Strand	Frame
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Query  42   ATGTTCCTTTTTTGCATTTATTGCGATTCTTTCTTCATGAATATCATAATTGTAATAGTC  101
Sbjct  1    ............................................................  60

Query  102  TTCTCATTACTCAGAACAAATCTATTTATGTTTTTTCAAATGAAAATAAAAGACTATTTC  161
Sbjct  61   ............................................................  120

Query  162  AGTTACTATACAATTCTTATGCTTTTGAATGTGAATTTTTATTAGttttttttCGTAAAC  221
Sbjct  121  ............................................................  180

Query  222  AATCTTATTATTTACGATTAACATCTTCTGCAACTTTTCTTGAACGAACCCATTTCTATA  281
Sbjct  181  ............................................................  240

Query  282  GAAAAATAGAACATCTTCGAATAGAACATTTTTTCGTAGTATGTCGTAACTATTTTCATA  341
Sbjct  241  ............................................................  300

Query  342  GAACTCGATGGTTCTTCAAAAATCCTTTCATGCATTATGTTCGATATCAAAGAAAGGCAA  401
Sbjct  301  ............................................................  360

Query  402  TTGTTGCTTCAAGGGGGACTCATTTTCTGATGAAGAAATGGAAATCCCATTTTGTCAATT  461
Sbjct  361  ............................................................  420

Query  462  TCTGGCAATATTATTTTCGCTTTTGGTCTCGACCGTACAGAATTCATATAAATCATTTAT  521
Sbjct  421  ............................................................  480

Query  522  CAAACTATTCCTTCTATTTTCTAGGTTATTTTTCAAGTCTACTAATAAATTCTTCGGCGG  581
Sbjct  481  ............................................................  540

Query  582  TAAGGAATCAAATGTTAGAGAATTCATTTCTAATGGATACCGTTACTAAGAAATTTGATA  641
Sbjct  541  ............................................................  600

Query  642  CCATAGTCCCAGTTATTCTTCTTATTGAATCCTTGTCTAAAGCTAAATTTTGTACCGTAT  701
Sbjct  601  ............................................................  660

Query  702  CAGGCCATCCTATTAGTAAGCCGATCTGGGCCGATTTCTCAGATTCTGATATTATTGATC  761
Sbjct  661  ..........................T.................................T.........  720

Query  762  GATTTGGTC-GATATGTAGAAATCTTTCTCATTATCACAGCGGATCCTCaaaaaaaCAA  820
Sbjct  721  .........G.....................................................-..-  778

Query  821  GGGAttt  827
Sbjct  779  ..-....  784
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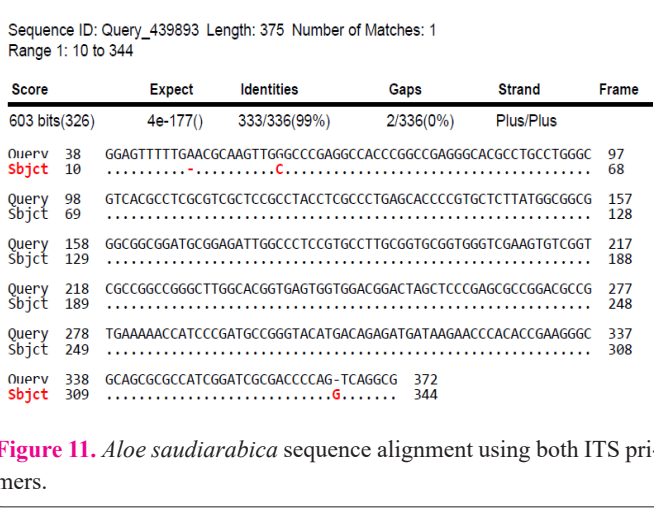
Figure 10. *Aloe saudiarabica* sequence alignment using both *matK* primers.


Sequence ID: Query_439893 Length: 375 Number of Matches: 1
Range 1: 10 to 344

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Sbjct  10   ..........-..........C.....................................  68

Query  98   GTCACGCCTCGCGTCGCTCCGCCTACCTCGCCCTGAGCACCCCGTGCTCTTATGGCGGCG  157
Sbjct  69   ............................................................  128

Query  158  GGCGGCGGATGCGGAGATTGGCCCTCCGTGCCTTGCGGTGCGGTGGGTCGAAGTGTCGGT  217
Sbjct  129  ............................................................  188

Query  218  CGCCGGCCGGGCTTGGCACGGTGAGTGGTGGACGGACTAGCTCCCGAGCGCCGGACGCCG  277
Sbjct  189  ............................................................  248

Query  278  TGAAAAACCATCCCGATGCCGGGTACATGACAGAGATGATAAGAACCCACACCGAAGGGC  337
Sbjct  249  ............................................................  308

Query  338  GCAGCGCGCCATCGGATCGCGACCCCAG-TCAGGCG  372
Sbjct  309  ..........................G.......  344
```

Figure 11. *Aloe saudiarabica* sequence alignment using both ITS primers.

Table 10. A brief overview of the species that are most similar to *Aloe saudiarabica* as identified by BOLD and GenBank.

	***matK* (first primers)**	***matK* (second primers)**	**ITS1**	**ITS2**
GenBank	*Aloe vera*	*Aloe vera*	*Aloe retrospiciens*	*Aloe vera*
BOLD	*Aloe vera*	*Aloe vera*	*Aloe vera*	*Aloe haemanthifolia*

Discussion

The presence and distribution of rare plant species hold paramount significance in preserving the biodiversity of an ecosystem. A systematic documentation of these species can provide crucial information to direct conservation efforts, determine the priority of species and habitats that require protection, and advance our comprehension of plant evolution, ecology, and systematic studies (16,17). The best approach to plant identification and documentation is to explore some of the plant's unique genetic markers. One of the most famous genetic markers on which multiple studies have been conducted is the plastid genes (*rbcL* and *matK*). The *matK* previously showed better evidence than its predecessor, *rbcL*, since it contains more diverse regions (18, 19). In addition, the trend has recently become more directed towards the sequences of ITS also because they contain distinctive regions and are useful for distinguishing between plants (20). Accordingly, and due to the importance of documenting rare plants, this study was designed to explore some genetic markers to distinguish *A. saudiarabica* plant. The study, which is considered the first of its kind to document this rare plant, included exploration of the genes of *rbcL* and *matK* along with the sequences of ITS. The current study showed the weakness of the possibility of using only *rbcL* as a genetic marker for *A. saudiarabica* plant under study. Perhaps one of the reasons why DNA amplification did not work here was the lack of more specialized primers. However, it has been established that utilizing the *rbcL* gene as the sole criterion for distinguishing between plant species is inadequate (21). On the other hand, the rest of the genetic markers explored showed good results, as they fully confirmed the identification at the plant genus level. These tags included *matK* and ITS including each pair of primers used. And through the different databases that were used, the sequences of these markers showed that the plant under study is of the genus *Aloe*. In many other recent studies, in which these markers were also used, and through which the ability of these markers to distinguish between plant species appeared with high efficiency (22, 23). The results of the exploration through the *matK* genetic marker and using different primers showed that the identification was successful, as the genus *Aloe*, and especially the *A. vera* species, was the closest, perhaps, to the availability of the total identification of the genome of this plant. Confirmation, on the other hand, only one sequence of the *matK* was got as a superposition of the two sequences obtained through primer pairs. By comparing this last superimposed sequence, which certainly contains the common part between the two original sequences, the same results appeared in the definition process. In this way, the efficiency of *matK* was confirmed as a successful marker for distinguishing *A. saudiarabica* plant. This is fully supported by the fact that the *matK* gene always contains regions of divergence in sequences that are characteristic of different species of plants (24, 25). On the other hand, the exploration of the sequences of the ITS2 region obtained with the pairs of primers for ITS2, whether original or the last superimposed, showed good results, as it succeeded in detecting the plant completely. The strength of these sequences and their containment of many areas of difference appeared here, as the *A.* species closest to *A. saudiarabica* here had some more diversity than its predecessor in the case of *matK* gene. Also, the results showed that the pairs of primers ITS1 were not sufficiently specific like ITS2, which often in the first attempts do not give good results, while the trend here is the latest and closest to use and distinguish plant species is through the regions of ITS2 (26, 27). In conclusion, it can be said that among the genetic markers on which the study was conducted, the efficiency of the *matK* and ITS2 sequences appeared to distinguish *A. saudiarabica* plant, and therefore the study recommends using them to document this rare plant. The study also recommends an exploration of the sequences of *rbcL* and ITS1, but with more specialized designed primers. Finally, this study is considered the first of its kind in attempts to explore the distinctive genetic markers of *A. saudiarabica* plant and showed promising results in this regard.

Conflict of Interest

The author declares that there is no conflict of interest.

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