Original article

Molecular identification and phylogenetic analysis of *Aloe shadensis* from Saudi Arabia based on *matK*, *rbcL* and ITS DNA barcode sequence

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**A B S T R A C T**

The Kingdom of Saudi Arabia thrives with great plant diversity, including rare plants of the family Asphodelaceae that have multiple benefits and are still being studied. *Aloe shadensis* is one of these plants that must be preserved and documented in its natural environment. The most appropriate molecular approach currently approved for documentation is the sequencing of some genomic markers. The current study is the first to use genomic markers to record this rare plant. In this study, the plastid genes *matK* (Maturase K), *rbcL* (Ribulose-bisphosphate carboxylase/oxygenase large subunit), and the nuclear region ITS (Internal transcribed spacer) were used to reveal their efficiency in identifying the plant under study. This study is the first to deal with this plant and document it using these genetic markers. The study showed a promising result concerning identifying the sequence of the *matK* gene and ITS region, while the *rbcL* gene did not give a good indicator through the used primers. The obtained sequences of the *matK* gene and the ITS region were determined through two different sets of primers in each case then deposited in GenBank. The evolutionary relatedness of *Aloe shadensis* was established with the different species of *Aloe*. The study showed that the closest species is *Aloe vera* with a similarity of more than 99 %. The study concludes with the possibility of using these genes to correctly identify, distinguish and document the species of *Aloe shadensis*.

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1. Introduction

Kingdom of Saudi Arabia possesses enormous plant diversity. Their benefits are not yet fully discovered and hence the need to conserve them. These plants include the family Asphodelaceae and the genus *Aloe*, in particular, some vulnerable species. Therefore, there is a need to document such plants genetically and their phenotypic characteristics to attain the ideal identification of the species (Das et al., 2015). Such studies are of great importance for understanding the characteristics of these species as well as the whole family (Jaiswal et al., 2021). *Aloe shadensis* is distinguished by its presence in Jabal Shada in Al-Baha region, Saudi Arabia only and distinctive phenotypic characteristics. Many of its characteristics and naming are mentioned in several sources (Chaudhary, 2001; Carter et al., 2011). This study is the first to document this plant genetically. Barcoding is based on determining preserved DNA regions in different species (Wattoo et al., 2016). Many genes were reported useful since the development of DNA barcoding of plants in 2008 (Yu et al., 2021). The most common markers in previous studies include the plastid *matK* and *rbcL*, the nuclear ITS1, and ITS2. Moreover, the combination of these markers is important to confer the species identification correctly (Techen et al., 2014; Ganie et al., 2015; Kress, 2017). The study aimed to evaluate *rbcL*, *matK*, and ITS genetic markers in DNA barcoding of *Aloe shadensis* and provide recommendations about the efficacy in identifying this species properly.

2. Materials and methods

2.1. Plant sampling

The plant samples have collected in 2020 from Jabal Shada area with the coordinates of the sites, Latitude, 19.8541 and Longitude, 41.3110, in Al-Baha, KSA. The plant was recognized by its morphological features and has been confirmed. The plant leaf was used to conduct the study (Fig. 1).
2.2. Molecular identification

2.2.1. DNA extraction

DNA has been extracted from the plant leaf sample (100 mg) following the kits’ manual (NucleoSpin Plant II, Macherey-Nagel). The purity of the extracted DNA was ensured by gel electrophoresis and documentation (Bio-Rad).

![Fig. 1. The used leaves of Aloe shadensis for DNA extraction.](image)

### Table 1

| Loci       | Primers | Direction | Sequence                  | Reference                  |
|------------|---------|-----------|---------------------------|----------------------------|
| rbcl       | rbcl.1F | F         | 5’-ATGTCACCACAACAGAAAC-3’ | Lledo et al. (1998)        |
|            | rbcl.724R | R         | 5’-TCCCATGTACAGGAGACCC-3’ |                            |
| matK       | matK-XF | 1st       | 5’-TAATTTATACCTGAATC-3’   | Dunning and Savolainen (2010) |
|            | matK-MALP-R1 | 2nd | 5’-ACAAAGAAATCCAGATAT-3’  |                            |
| ITS 1      | ITS 5a  | F         | 5’-CCTTATCATTTAGAGGAAGGAG-3’ | Stanford et al. (2000)     |
|            | ITS 4   | R         | 5’-TCTTCCGATTATGATGCT-3’  |                            |
| ITS 2      | ITS S2F | F         | 5’-ATGCGAATCTTGGTCAATG-3’  | Chio et al. (2007)         |
|            | ITS S3R | R         | 5’-GACGCTTCTCCAGACTAAG-3’  |                            |

### Table 2

The plant under study, Aloe shadensis, and the percentage of similarity with the closest species in the GenBank based on the matK gene sequence by the first pair of primers.

| No. | Species description       | Scientific name | Aligned sequence (bp) | Coverage (%) | E value | Similarity (%) | Accession       |
|-----|---------------------------|-----------------|-----------------------|--------------|--------|----------------|-----------------|
| 1   | Aloe shadensis            | Aloe shadensis  | 803                   | 100          | 0      | 100            | MZ458425        |
| 2   | Aloe vera voucher Aloe vera | Aloe vera      | 800                   | 99           | 0      | 99.88          | KX377524.1      |
| 3   | Aloe vera CMFR8774        | Aloe vera      | 800                   | 99           | 0      | 99.88          | KY556640.1      |
| 4   | Aloe vera                 | Aloe vera      | 800                   | 99           | 0      | 99.98          | JQ276402.1      |
| 5   | Aloe vera isolate 2       | Aloe vera      | 799                   | 99           | 0      | 99.75          | FJ237261.2      |
| 6   | Aloe vera voucher Av3     | Aloe vera      | 795                   | 99           | 0      | 99.87          | KP072727.1      |
| 7   | Aloe vera                 | Aloe vera      | 795                   | 99           | 0      | 99.87          | MW176075.1      |
| 8   | Aloe vera voucher Av1     | Aloe vera      | 794                   | 99           | 0      | 99.75          | KP072725.1      |
| 9   | Aloe vera                 | Aloe vera      | 798                   | 99           | 0      | 99.50          | AY3323721.1     |
| 10  | Aloe compressa var. compressa | Aloe compressa var. compressa | 799 | 99 | 0 | 99.50 | AY3323721.1 |
| 11  | Aloe gneissicola          | Aloe gneissicola | 799             | 99           | 0      | 99.50          | AY3323720.1     |

### Table 3

The maximum likelihood estimate of substitution matrix in the constructed phylogenetic tree of Aloe shadensis matK gene sequence by the first pair of primers.

| From | To   | A   | T   | C   | G   |
|------|------|-----|-----|-----|-----|
| A    |      | 7.5782 | 3.2754 | 3.2754 | 9.3227 |
| T    | 6.0101 | -    | 9.5153 | -    | 2.8379 |
| C    | 6.0101 | 22.0155 | -    | -    | 2.8379 |
| G    | 19.7435 | 7.5782 | 3.2754 | -    | -    |

2.2. Molecular identification

2.2.1. DNA extraction

DNA has been extracted from the plant leaf sample (100 mg) following the kits’ manual (NucleoSpin Plant II, Macherey-Nagel). The purity of the extracted DNA was ensured by gel electrophoresis and documentation (Bio-Rad).

![Fig. 2. Phylogenetic tree by neighbor-joining of Aloe shadensis with the closest species based on the matK gene sequence by the first pair of primers.](image)
The plant under study, Aloe shadensis, and the percentage of similarity with the closest species in the GenBank based on the matK gene sequence by the second pair of primers.

| No. | Species description | Scientific name | Aligned sequence (bp) | Coverage (%) | E value | Similarity (%) | Accession |
|-----|---------------------|-----------------|-----------------------|--------------|---------|----------------|-----------|
| 1   | Aloe shadensis      | Aloe shadensis  | 825                   | 100          | 0       | 100            | MZ438002  |
| 2   | Aloe vera voucher   | Aloe vera       | 800                   | 100          | 0       | 99.76          | KX377524.1|
| 3   | Aloe vera isolate   | Aloe vera       | 837                   | 100          | 0       | 99.64          | AY323726.1|
| 4   | Aloe compressa      | Aloe compressa  | 838                   | 100          | 0       | 99.40          | AY323721.1|
| 5   | Aloe gneissicola    | Aloe gneissicola| 837                   | 100          | 0       | 99.40          | AY323720.1|
| 6   | Aloe purpurea WV    | Aloe purpurea   | 838                   | 100          | 0       | 99.40          | KX270418.1|
| 7   | Aloe purpurea MAU   | Aloe purpurea   | 838                   | 100          | 0       | 99.40          | KX270416.1|
| 8   | Aloe macra voucher  | Aloe macra      | 838                   | 100          | 0       | 99.40          | KX270415.1|
| 9   | Aloe deserti voucher| Aloe deserti    | 838                   | 100          | 0       | 99.40          | KU748261.1|
| 10  | Aloe nyeriensis     | Aloe nyeriensis | 838                   | 100          | 0       | 99.40          | KU748259.1|

Fig. 3. The Sequence alignment of Aloe shadensis (Query) and the first next species Aloe vera (Subject) based on matK gene sequence by the first pair of primers.

Fig. 4. Phylogenetic tree by neighbor-joining of Aloe shadensis with the closest species based on the matK gene sequence by the second pair of primers.
2.2.2. Amplification

The primers used in the amplification are listed in Table 1. Master Mix Phire Plant (Thermo Scientific) was used. The reaction mixture and cycling instructions were followed as described. GeneAmp cycler 9700 was used for amplification (Applied Biosystems). The PCR product was subjected to ExoSAP-IT (GE-Healthcare) protocol for cleanup. Before sequencing, the purity of the PCR product was ensured by gel electrophoresis (1.2% agarose). A 100 bp DNA marker (Bioatlas) was used as a molecular standard. A Bio-Rad documentation system has been used to visualize the gel under UV light.

2.2.3. Sequencing

Sequencing was done following the kit’s protocol (BigDye Terminator v3.1, Applied Biosystems). The same PCR primers have been used for sequencing. The reaction has done in An ABI-3500 DNA-Analyzer (Applied Biosystems).

2.2.4. Data analysis

The assembled sequences were used to classify the plant. This was conducted through the GenBank BLAST tool and the BOLD database systems. The final sequences have then been deposited in GenBank. Phylogenetic analysis has been carried out by MEGA-X software (Kumar et al., 2018).

3. Results

The results showed that the primers used in the amplification process for the genetic markers under study mentioned in Table 1 gave fragments of molecular weights as expected, except for the rbcL gene, and the result was negative, and therefore its sequence was not determined. The rest of the markers were positive, as in the order of Table 1 the matK gene, followed by the ITS region, using two pairs of primers in each case. The molecular weights of

Table 5

The maximum likelihood estimate of substitution matrix in the constructed phylogenetic tree of Aloe shadensis matK gene sequence by the second pair of primers.

| From/To | A   | T   | C   | G   |
|---------|-----|-----|-----|-----|
| A       | –   | 8.7625 | 3.8645 | 8.7967 |
| T       | 7.0988 | –   | 8.1423 | 3.4057 |
| C       | 7.0988 | 18.4623 | –   | 3.4057 |
| G       | 18.3357 | 8.7625 | 3.8645 | –   |

Fig. 5. The Sequence alignment of Aloe shadensis (Query) and the first next species Aloe vera (Subject) based on matK gene sequence by the second pair of primers.

Table 6

The plant under study, Aloe shadensis, and the percentage of similarity with the closest species in the GenBank based on the ITS region sequence by the ITS1 pair of primers.

| No. | Species description | Scientific name | Aligned sequence (bp) | Coverage (%) | E value | Similarity (%) | Accession |
|-----|---------------------|-----------------|-----------------------|-------------|---------|----------------|-----------|
| 1   | Aloe shadensis      | Aloe shadensis  | 404                   | 100         | 0       | 100            | MZ420214  |
| 2   | Aloe retrosicpeis voucher Grace163 | Aloe retrosicpeis | 367                   | 90          | 0       | 98.91          | KJ557911.1 |
| 3   | Aloe dorotheae voucher Grace202 | Aloe dorotheae | 367                   | 90          | 0       | 98.91          | KJ557867.1 |
| 4   | Aloe camperi voucher Grace153 | Aloe camperi | 367                   | 90          | 0       | 98.91          | KJ557857.1 |
| 5   | Aloe sinkatana voucher Grace135 | Aloe sinkatana | 367                   | 90          | 0       | 98.91          | KJ893738.1 |
| 6   | Aloe vera voucher Grace220 | Aloe vera | 366                   | 90          | 0       | 98.91          | KJ893746.1 |
| 7   | Aloe minima voucher Klopper & Abbot464 | Aloe minima | 367                   | 90          | 0       | 98.64          | KJ557469.1 |
| 8   | Aloe trichosantha voucher Grace151 | Aloe trichosantha | 366                   | 90          | 0       | 98.37          | KJ557922.1 |
| 9   | Aloe fleurentinorum voucher A.G.Miller&D.Long3459 | Aloe fleurentinorum | 368                   | 90          | 0       | 98.09          | KJ557872.1 |
| 10  | Aloe ankoberensis voucher Grace148 | Aloe ankoberensis | 367                   | 90          | 0       | 98.09          | KJ557848.1 |
| 11  | Aloe thorncroftii isolate P7710 | Aloe thorncroftii | 357                   | 88          | 0       | 98.88          | KF013327.1 |
matK amplicon by first and second pair were 900 and 850, while the weights for the ITS region were 800 and 500 using ITS1 and ITS2 primers, respectively. The partial sequences of these genetic markers were obtained using the same primers, then four assembled sequences were obtained and deposited in the GenBank. The sequences were used through the BOLD system to identify the plant, and also the closest species in the GenBank were obtained. The phylogenetic trees were established, and the plant was compared in each case with the neighboring species. Aloe shadensis was deposited and documented here in this study with genetic markers for the first time. The results showed that through any identification system and for all the markers used, the plant genus is Aloe. Aloe vera was the most closely related species with greater extent than the other species of Aloe. Below are the results of each genetic marker used in order. The sequence alignment was performed in the MEGAX program using ClustalW, and evolution trees were established with Bootstrap 1000 replicates. In the case of the matK sequence (803 bp) using the first pair of primers (matK-XF and matK-MALP-R1) and using the BOLD system for identification, the closest species was Aloe vera with a percentage of 99.88%. By using the BLAST tool in the gene bank, it was also shown that the closest species is Aloe vera with the same percentage of 99.88% (Table 2). The phylogenetic tree for this sequence was constructed.

Table 7
The maximum likelihood estimate of substitution matrix in the constructed phylogenetic tree of Aloe shadensis ITS region sequence by the ITS1 pair of primers.

| From| To  | A     | T     | C     | G     |
|-----|-----|-------|-------|-------|-------|
| A   | 5.8594 | 11.6306 | 9.5684 |
| T   | 9.1819 | 12.0895 |
| C   | 4.6258 | 11.6306 | 12.0895 |
| G   | 4.3518 | 9.5684 | 11.6306 |

matK amplicon by first and second pair were 900 and 850, while the weights for the ITS region were 800 and 500 using ITS1 and ITS2 primers, respectively. The partial sequences of these genetic markers were obtained using the same primers, then four assembled sequences were obtained and deposited in the GenBank. The sequences were used through the BOLD system to identify the plant, and also the closest species in the GenBank were obtained. The phylogenetic trees were established, and the plant was compared in each case with the neighboring species. Aloe shadensis was deposited and documented here in this study with genetic markers for the first time. The results showed that through any identification system and for all the markers used, the plant genus is Aloe. Aloe vera was the most closely related species with greater extent than the other species of Aloe. Below are the results of each genetic marker used in order. The sequence alignment was performed in the MEGAX program using ClustalW, and evolution trees were established with Bootstrap 1000 replicates. In the case of the matK sequence (803 bp) using the first pair of primers (matK-XF and matK-MALP-R1) and using the BOLD system for identification, the closest species was Aloe vera with a percentage of 99.88%. By using the BLAST tool in the gene bank, it was also shown that the closest species is Aloe vera with the same percentage of 99.88% (Table 2). The phylogenetic tree for this sequence was constructed.

Fig. 6. Phylogenetic tree by neighbor-joining of Aloe shadensis with the closest species based on the ITS region sequence by the ITS1 pair of primers.

Fig. 7. The Sequence alignment of Aloe shadensis (Query) and the first next species Aloe retropisciens (Subject) based on ITS region sequence by the ITS1 pair of primers.
with the closest species (Fig. 2) with the estimated substitution matrix (Table 3). The overall mean distance amongst Aloe shadensis and the individuals represented in the phylogenetic tree was 0.1 with a standard error (S.E. equals 0.0). Fig. 3 shows the alignment of the sequence of Aloe shadensis and its closest species, Aloe vera. In the case of the matK sequence (825 bp) using the second pair of primers (1R_ kim and 3F_ kim) and using the BOLD system for identification, the closest species was Aloe vera with a percentage of 99.76% (Table 4). The phylogenetic tree for this sequence was constructed with the closest species (Fig. 4) with the estimated substitution matrix (Table 5). The overall mean distance amongst Aloe shadensis and the individuals represented in the phylogenetic tree was 0.25 with a standard error (S.E. equals 0.0). Fig. 5 shows the alignment of the sequence of Aloe shadensis and its closest species, Aloe vera. In the case of the ITS region sequence (404 bp) using the ITS1 pair of primers (ITS 5a and ITS 4) and using the BOLD system for identification, the closest species was Aloe sinkatana with a percentage of 99.69% (Table 6). The phylogenetic tree for this sequence was constructed with the closest species (Fig. 6) with the estimated substitution matrix (Table 7). The overall mean distance amongst Aloe shadensis and the individuals represented in the phylogenetic tree was 0.13 with a standard error (S.E. equals 0.0). Fig. 7 shows the alignment of the sequence of Aloe shadensis and its closest species, Aloe retropliciens. In the case of the ITS region sequence (386 bp) using the ITS2 pair of primers (ITS S2F and ITS S3R) and using the BOLD system for identification, the closest species was Aloe sinkatana with a percentage of 100%. By using the BLAST tool in the gene bank, it was also shown that the closest species is Aloe vera with a percentage of 99.73% (Table 8). The phylogenetic tree for this sequence was constructed with the closest species (Fig. 8) with the estimated substitution matrix (Table 9). The overall mean distance amongst Aloe shadensis and the individuals represented in the phylogenetic tree was 0.43 with a standard error (S.E. equals 0.01). Fig. 9 shows the alignment of the sequence of Aloe shadensis and its closest species, Aloe vera. Table 10 shows a summary of the closest species to Aloe Shadensis using the two identification systems. An Alignment of both sequences of the matK gene was performed using both pairs of primers, and a sequence of 777 bp was obtained (Fig. 10). Another alignment of the two ITS region sequences was also performed, and a sequence of 332 bp was obtained (Fig. 11). By matching the final matK sequence and also using both identification systems, the results showed that Aloe vera is the closest species to Aloe shadensis, with a percentage of more than 99%. Also, the ITS region gave a similarity of more than 99 % to Aloe vera in the GenBank system. In the BOLD system, Aloe dorothaeae was given, followed by other species of Aloe, then Aloe vera ranked 89th. In all these species, the sequence covering ratio has decreased from 322 in the first species to 194 in the case of Aloe vera.

### Table 8

| No. | Species description | Scientific name | Aligned sequence (bp) | Coverage (%) | E value | Similarity (%) | Accession |
|-----|---------------------|-----------------|-----------------------|-------------|---------|----------------|-----------|
| 1   | Aloe shadensis      | Aloe shadensis  | 386                   | 100         | 0       | 100            | MZ424222  |
| 2   | Aloe vera voucher B0709 | Aloe vera   | 365                   | 94          | 0       | 99.73          | MN519271.1|
| 3   | Aloe vera bio-material AHP_Lot_147 | Aloe vera   | 364                   | 94          | 0       | 99.45          | MK087867.1|
| 4   | Aloe nyeriensis voucher NMKEA 13614 | Aloe nyeriensis | 365                   | 94          | 0       | 98.90          | MT137508.1|
| 5   | Aloe kedongensis voucher NMKEA 13642 | Aloe kedongensis | 365                   | 94          | 0       | 98.63          | MT137515.1|
| 6   | Aloe tormentorii isolate ALT02 | Aloe tormentorii | 357                   | 92          | 0       | 98.60          | KX689271.1|
| 7   | Aloe volkensii voucher NMKEA 13619 | Aloe volkensii | 364                   | 94          | 0       | 98.36          | MT137509.1|
| 8   | Aloe tormentorii isolate ALT01 | Aloe tormentorii | 354                   | 91          | 0       | 98.31          | KX689270.1|
| 9   | Aloe retropliciens voucher Grace163 | Aloe retropliciens | 327                   | 84         | 2.00E-171 | 99.69          | KJ557911.1|
| 10  | Aloe dorotheae voucher Grace202 | Aloe dorotheae | 327                   | 84         | 2.00E-171 | 99.69          | KJ557867.1|
| 11  | Aloe camperi voucher Grace153 | Aloe camperi | 327                   | 84         | 2.00E-171 | 99.69          | KJ557857.1|

### Table 9

The maximum likelihood estimate of substitution matrix in the constructed phylogenetic tree of Aloe shadensis ITS region sequence by the ITS2 pair of primers.

| From|To |A  | T  | C  | G  |
|-----|---|---|----|----|----|
| A   | -  | 2.7125 | 5.8786 | 6.3531 |
| T   | 3.1065 | -  | 37.4319 | 6.1764 |
| C   | 3.1065 | 17.2718 | -  | 6.1764 |
| G   | 3.1954 | 2.7125 | 5.8786 | -  |
4. Discussion

Combining coding and noncoding genetic markers is the best approach to DNA barcoding of plants. The most commonly investigated markers in many studies are the plastid conserved rbcL gene and the more variable matK gene. Recently, the (ITS) region also has proven a useful variable marker (Kress, 2017). So, this study was planned to investigate the efficiency of these markers in the
The remainder markers, including the matK gene and the ITS region, were partially sequenced by the same primers used in amplification in each case. The results confirmed the identity of the plant genus, Aloe. In many studies, the matK gene and the ITS region genetic markers have been proved very efficient in distinguishing the plant species and hence being promise candidates in the barcoding of plants (Han et al., 2016; Yu et al., 2021). The sequence of the matK gene using both pairs of primers showed effectiveness in identifying the species, as many species of Aloe were found similar to it, although the closest was Aloe vera, due to the complete sequencing of its genome. Also, by performing the alignment of the two sequences of Aloe shadensis and obtaining a common sequence with high identity, it gave the same results in the identification, which supports its use as a distinguishing marker of the Aloe shadensis plant. The matK gene often contains many variable pieces and is considered a distinctive marker for the differentiation of plant species (Dong et al., 2012). In the case of the ITS region, the sequences obtained using the ITS1 and the ITS2 primers and also their alignment revealed some variations concerning Aloe species relatedness to Aloe shadensis. This was also another support to the power of species discrimination through this region. The successful ITS2 region sequence was achieved by the two different sets of primers. The sequence of the ITS1 region was not represented here equally well as in the case of the ITS2 region sequence. Usually, the ITS1 region may encounter a little difficulty in sequencing despite the successful amplification, and recently, the ITS2 region has been paid more attention (Wang et al., 2016; Yu et al., 2021). In conclusion, the study proved the efficiency of the matK gene and the ITS2 region as reliable markers in the barcoding of Aloe shadensis. Even though the results of the rbcL gene and the ITS1 region were not very promising, they can give better results with more designed specific primers. The study recommends the importance of using these genetic markers in documenting this rare plant and conducting further studies on this subject.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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