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DNA Barcoding of *Ricinus communis* from Different Geographical Origin by Using Chloroplast *matK* and Internal Transcribed Spacers

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ABSTRACT

Ricinus communis have attracted considerable attention because of its specific industrial and pharmacological activities. DNA barcodes can be used as reliable tools to facilitate the identification of medicinal plants for the safe use, quality control and forensic investigation. In this study, the differential identification of eight accessions of *R. communis* was investigated through DNA sequence analysis of two candidate DNA barcodes. The nucleotide sequence of internal transcribed spacers (ITS2) and chloroplast maturase gene (*matK*) have been determined to construct the phylogenetic tree. The phylogenetic relationships of accessions based on the nrITS2 region and partial *matK* region showed that all accessions in this study were related to three geographical origins. Based on sequence alignment and phylogenetic analyses we concluded that the ITS2 sequences can distinguish *R. communis* accessions from different geographical distributions.

Keywords: DNA Barcoding; Internal Transcribed Spacer; Maturase K; *Ricinus communis*

1. Introduction

The castor oil plant, *Ricinus communis* also known as Palma(e) christi or wonder tree. It is a perennial scrub of the family Euphorbiaceae. The plant can vary greatly in its growth habit and morphology. The variability has been increased by breeders who have selected a range of cultivars for use as ornamental plants, and for commercial production of castor oil [1]. The castor oil is a wonderful universal remedy for a large number of health concerns. The oil has been used as for warts, cold tumors, indurations of the abdominal organs, lacteal tumors and indurations of the mammary gland [2]. Seeds have high oil content, with multiple industrial applications such as paints, lubricants, cosmetics, polymers and biofuels [3]. DNA barcoding is a method of describing and identifying species by analyzing sequence information from one or a few short standardized loci amplified with universal primers. To standardize the international use of DNA barcodes, the scientific community has made considerable efforts searching for suitable DNA regions to barcode every species [4-8]. DNA barcoding provides a rapid identification tool, utilizing only minute amount of tissue from any stage of development of a plant or animal. DNA barcodes can be used to identify specimens correctly, to expand the discovery of new species, in tackling illegal trade of endangered species of both plant and animals and in forensic investigation to help detect poisonous materials in life-threatening cases [9,10]. DNA barcoding has been proposed as a novel and powerful taxonomic tool [6,11], the mitochondrial cytochrome oxidase subunit I (CO1) is a widely used barcode in a range of animal groups [12-15] this locus is unsuitable for use in plants due to its low mutation rate [4,14,15]. A variety of loci have been suggested as DNA barcodes for plants, including coding genes in plastid genome and the multicopy nuclear Internal Transcribed Spacer (ITS) are two of the leading candidates [4]. Thus, this issue is addressed in the present study by comparing the feasibility of using each of these proposed DNA barcodes (*matK*, ITS1, ITS2) to identify genetic variations of *R. communis* accessions from different geographical distributions.

2. Materials and Methods

2.1. Plant Materials

Eight accessions from *R. communis* were examined. The

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eight accessions are as follows: R1 (Kadiogo), R3 (Sour), R5 (Tunisia), R7 (Venezuela), R8 (Yemen, Hardamout), and R9 (Yemen, Lawdar), were obtained from the Millennium Seed Bank, Kew Royal Botanic Gardens, however, R2 and R6 were collected from Egypt and United Arab Emirates (UAE), respectively. The plant specimens used in this study are summarized in **Table 1**.

2.2. DNA Isolation

Plant seeds are hard to lyse due to seed coat and hard cotyledon inside, the seed coats mainly contain tannins that inhibit PCR. The inner seed material contains starches and lipids that can foam and make lysis difficult [16]. The seeds of each accession were immersed in liquid nitrogen and crushed using sterile mortar and pestle to get a fine powder. An automatic DNA extraction (Maxwell[16], Promega) and DNeasy plant mini kit (Qiagen) were used for DNA extraction. DNeasy plant mini kit with slight modification in which 0.4% (v/v) *β*-mercaptoethanol was added to AP1/E lysis buffer was performed. Quality of the extracted DNA was determined using gel electrophoresis.

2.3. PCR Amplification

A total volume of 30 µL of PCR reaction mixture contained the following: 15 µL of PCR Master Mix (Qiagen, Germany), giving a final concentration of 200 mM each deoxynucleotide and 1.5 mM $MgCl_2$, 20 pM each primer (**Table 2**), 2 µL of genomic DNA (50 ng) and the rest was adjusted with sterile distilled water. PCR amplification was performed with a thermal cycler (T_{100}, BIORAD) as follows: one cycle at 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 s, 55˚C for 30 s and 72˚C for 1 min, followed by an elongation step at 72˚C for 5 min. All the PCR conditions were the same for all the primer pairs.

2.4. Agarose Gel Electrophoresis

PCR products were examined using 1.5% agarose gel electrophoresis in 1X TBE buffer at 70 V for ~45 min. Gel images were obtained using Gel documentation (Major Bioscience, Taiwan) imaging system. The size of PCR products resulting from the primer pairs of the specific barcoding gene were determined by using a 50 bp sharp mass (Euro Clone, Italy) and 100 bp DNA ladder (Promega, Madison).

2.5. Sequencing and Alignment

PCR products sent to the Source Bioscience (Nottingham, UK) for sequencing after simple purification and sequenced according to the method originally described by Sanger [17]. ITS2 and *matK* products were sequenced using the same primer pairs as used for the initial amplification. The sequences from each DNA region were aligned by CLUSTALW and genetic distance was computed using the MEGA 5.0 Kimura two-parameter (K2P) model [18]. The nucleotide sequence data of the partial *matK* sequence and ITS2 spacer were deposited in the Genbank nucleotide sequence databases with the accession numbers reported in **Table 2**. The phylogenetic trees were constructed using maximum likelihood (ML) in MEGA 5.0 software program. Bootstrap testing of 1000 replicates was performed to estimate the confidence level of the topology of the consensus tree.

3. Results

Our study showed that DNA extraction using Plant DNeasy minikit provided better yield and quality compared with automatic DNA extraction method that failed to produce higher quality and PCR amplification. For the ITS and *matK*, all samples showed an equal size of the PCR product. Excluding the primer flanking sites, the sizes of the ITS1, ITS2 and *matK* of all accessions were 360 to 440 and 790 bp in length, respectively as shown in **Figure 1**. The ITS1, ITS2 spacers and *matK* gene of all accessions were successfully amplified and only ITS2 and *matK* regions were successfully sequenced. For a

Table 1. Plant samples used in this study.

Sample ID	Geographical origin	Date of collection	Collector and MSB serial No.	Accession number	
				ITS2	*matK*
R1	BURKINA FASO, Kadiogo	1998	No.125723*	JX084257	JX084265
R2	Egypt, Zagazig	2009	F. Nael	JX084258	JX084266
R3	LEBANON, Sour	1998	No.129329*	JX084259	JX084267
R5	TUNISIA, Tataouine	1997	No.119694*	JX084260	JX084268
R6	UAE, Al-Ain	2009	F. Nael	JX084261	JX084269
R7	VENEZUELA, Nueva Esparta	1994	No.103716*	JX084262	JX084270
R8	YEMEN, Hadramout	1997	No.118664*	JX084263	JX084271
R9	YEMEN, Lawdar	1997	No.118387*	JX084264	JX084272

*The number is the serial number in the Millennium Seed Bank (MBS), Kew Royal Botanic Gardens.

Table 2. Universal primers for the amplification and sequencing of DNA barcodes.

Locus	Primer name	Primer sequence (5'-3')	References
ITS	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	White *et al.*, 1990
	ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	White *et al.*, 1990
	ITS3	5'-GCATCGATGAAGAACGCAGC-3'	White *et al.*, 1990
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White *et al.*, 1990
matK	matK472F	5'-CCCRTYCATCTGGAAATCTTGGTTC-3'	Yu *et al.*, 2011
	matK1248R	5'-GCTRTRATAATGAGAAAGATTTCTGC-3'	Yu *et al.*, 2011

ITS: Internal transcribed spacer; *matK*: Maturase K gene.

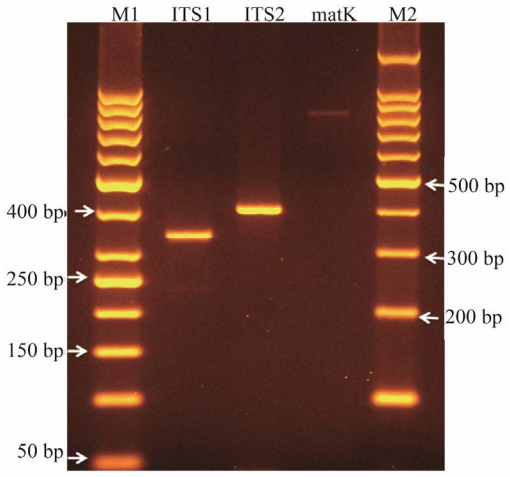

Figure 1. Agarose gel of electrophoresis of PCR products of ITS1, ITS2 and *matK* genes show a single band in the electrophoresis profiles, corresponding to 360, 440, and 790 bp in length. M1: 50bp sharp mass ladder; M2: 100 bp DNA ladder.

DNA-based identification of *R. communis*, two candidate DNA barcode sequences were submitted to multiple sequence alignment (MSA).

The nucleotide sequence alignment of ITS2 barcode of R1 (Kadiogo), R3 (Sour), R5 (Tunisia), R6 (UAE), R7 (Venezuela), R8 (Hadramout) and R9 (Lawdar) had 2, 2, 3, 3, 2, 3, and 3 base substitutions in comparison with those R2 (Egypt), respectively as shown in **Figure 2**. The *matK* sequences alignment of 8 accessions revealed that the sequence of R1 (Kadiogo), R5 (Tunisia), R6 (UAE), R7 (Venezuela), R8 (Hadramout), R9 (Lawdar) had only one base substitutions. On the other hand, the sequence of R3 (Lebanon, Sour) had 16 base substitutions compared with the sequence of R2 (Egypt) as shown in **Figure 3**. The sequence divergence among eight accessions of *R. communis* from different geographical origin varied from 0.00% to 0.78% (**Table 3**). In contrast, *matK* sequence divergence among 8 accessions varied was from 0.13 % to 0.75% (**Table 4**). The phylogenetic tree constructed by the *matK* gene analysis suggested that the eight accessions were divided into two clusters. R2 (Egypt) belong to the same cluster with R1 (Kadiogo), R5 (Tunisia), R6 (UAE), R7 (Venezuela), R8 (Hadramout), and R9 (Lawdar), while R3 (Lebanon, Sour) separated into another cluster (**Figure 4**).This tree was incompatible with that constructed by ITS2 analysis suggested that R7 (Venezuela) and R2 (Egypt), and R1 (Kadiogo) were in one cluster, other accessions (R5, R8, R9, R6 and R3) in the second cluster which were divided into two subclusters in the phylogenetic tree (**Figure 5**).

4. Discussion

The castor oil plant *R. communis* is one of the oldest drugs known to man. The first mention of it as a laxative can be found in 3500 year-old Ancient Egyptian papyrus scrolls. The most promising plastid candidate maturase K [15,19] was tested, along with the nuclear locus internal transcribed spacer (ITS2), which is also a most important candidate for plant barcoding [4,20]. The ITS2 region was selected as a barcode candidate because ITS2 sequences are potential general phylogenetic markers and are widely used for phylogenetic constructions at both the genus and species levels [21,22]. As the ITS2 region is one of the most common regions used for phylogenetic analyses [23]. In our study, nrITS1 regions were amplified cleanly in 8 accessions but sequencing was unsuccessful. Chodon *et al.* [24] reported that one of potentially negative factor for sequencing nrITS is the presence of ply-G, poly-C, and poly-A repeats. In general the nrITS2 region is more length-conserved than nrITS1, making it a more predictable amplicon to work with [7]. Our research shows that a single region *matK* or ITS2 a portion, it was demonstrated that the sequence nucleotide variation can distinguish genetics divergence among *R. communis* from different geographical origin; this was supported by sequence alignment analyses. In previous studies, ITS2 has already been suggested as a suitable marker applicable for phylogenetic reconstruction in eukaryotes by many researchers [21,22,25]. The *matK* coding region is one of the most rapidly evolving regions in chloroplasts and shows a high level of species discrimination among angiosperms, a fragment of 600 - 800 bp is

Table 3. Pairwise genetic distance of *matK* barcode region.

	matkR9	matkR8	matkR7	matkR6	matkR5	matkR3	matkR2	matkR1
matkR9	-							
matkR8	0.67864	-						
matkR7	0.68620	0.72401	-					
matkR6	0.65595	0.71267	0.43289	-				
matkR5	0.72590	0.69376	0.72779	0.69943	-			
matkR3	0.13043	0.70132	0.69376	0.67297	0.75992	-		
matkR2	0.71456	0.69376	0.73724	0.70510	0.71456	0.72779	-	
matkR1	0.67675	0.68242	0.65217	0.21928	0.69565	0.69187	0.71645	-

Table 4. Pairwise genetic distance of ITS2 barcode region.

	ITS2R6	ITS2R1	ITS2R2	ITS2R3	ITS2R5	ITS2R7	ITS2R8	ITS2R9
ITS2R6	-							
ITS2R1	0.00906	-						
ITS2R2	0.71299	0.71601	-					
ITS2R3	0.74018	0.73716	0.78852	-				
ITS2R5	0.00604	0.00906	0.71299	0.74018	-			
ITS2R7	0.72205	0.72508	0.71299	0.73716	0.72205	-		
ITS2R8	0.00604	0.00906	0.71299	0.74018	0.00000	0.72205	-	
ITS2R9	0.00604	0.00906	0.71299	0.74018	0.00000	0.72205	0.00000	-

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R2 Egypt          1   ATCTAGTTTTTGAACGCAAGTTGCGCCCGAAGCCTTTCGGCCGAGGGCACGCCTGCCTGG  60
R6 UAE            1   ...G.......................................................  60
R1 Kadiogo        1   ...G.......................................................  60
R3 Sour           1   ...G.......................................................  60
R5 Tunisia        1   ...G.......................................................  60
R7 Venezuela      1   ...G.......................................................  60
R8 Hadramout      1   ...G.......................................................  60
R9 Lawdar         1   ...G.......................................................  60

R2 Egypt         62   GTGTCACGCAATCGTCGCCCCCAACCCTTTCGATACATCGAGAGGGGGGCGGATTATGTC  121
R6 UAE           62   ...........................................................G.  121
R1 Kadiogo       62   ............................................................  121
R3 Sour          62   ...........................................................G.  121
R5 Tunisia       62   ...........T................................................G.  121
R7 Venezuela     62   ............................................................  121
R8 Hadramout     62   ...........T................................................G.  121
R9 Lawdar        62   ...........T................................................G.  121

R2 Egypt        122   CTCCCGTGCGCCTCGTGCATGCGGTTGGCCTAAAAATTGAGTCCCCGGCGACTATCGCCA  181
R6 UAE          122   ............................................................  181
R1 Kadiogo      122   ..........................................T.................  181
R3 Sour         122   ............................................................  181
R5 Tunisia      122   ............................................................  181
R7 Venezuela    122   ..........................................T.................  181
R8 Hadramout    122   ............................................................  181
R9 Lawdar       122   ............................................................  181

R2 Egypt        182   CGGCAATCGGTGGTTGTAAGACTCTCTGAAACTGCCGTGCGCGCTCGTCTGCCAAGAGGG  241
R6 UAE          182   ..A.........................................................  241
R1 Kadiogo      182   ............................................................  241
R3 Sour         182   ............................................................  241
R5 Tunisia      182   ............................................................  241
R7 Venezuela    182   ............................................................  241
R8 Hadramout    182   ............................................................  241
R9 Lawdar       182   ............................................................  241
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Figure 2. Part aligned sequence of the ITS2 region of eight accessions of *R. communis*. The dots indicate that the base at that position in the specified sequence is the same as the base in the sequence written at the top of the compilation.


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matKR2 Egypt          1    GACTCTTTCTTCATGAGTATTGGAATTGGAACAGTTTTATTATTCCGAAAGAAATCAATT  59
matKR6 UAE            1    ............................................................  59
matKR1 Kadiogo        1    ............................................................  59
matKR3 Sour           1    .........G..............G..CC..T....T.............G......  59
matKR5 Tunisia        1    ............................................................  59
matKR7 Venezuela      1    ............................................................  59
matKR8 Hadramout      1    ............................................................  59
matKR9 Lawdar         1    ............................................................  59

matKR2 Egypt          60   TCTATTTTTACAAAAAGTAATCCAAGATTTTTCGTGTTCCTATATAATTCTCATGTATAT  119
matKR6 UAE            60   ............................................................  119
matKR1 Kadiogo        60   ............................................................  119
matKR3 Sour           60   ................................G.................A.A....  119
matKR5 Tunisia        60   ............................................................  119
matKR7 Venezuela      60   ............................................................  119
matKR8 Hadramout      60   ............................................................  119
matKR9 Lawdar         60   ............................................................  119

matKR2 Egypt          120  GAATATGAATCCCTCTTCTTTTTTCTCCGTAACCAATCCTTTCATTTACGATCAACATTT  179
matKR6 UAE            120  ............................................................  179
matKR1 Kadiogo        120  ............................................................  179
matKR3 Sour           120  ..............G....G....C.....................................  179
matKR5 Tunisia        120  ............................................................  179
matKR7 Venezuela      120  ............................................................  179
matKR8 Hadramout      120  ............................................................  179
matKR9 Lawdar         120  ............................................................  179


matKR2 Egypt          360  AAATATTACCTTGTCCATTTATGTCAATGTCATTTTTATGTGTGGTTTCAACCGGAAAAG  419
matKR6 UAE            359  ............................................................  418
matKR1 Kadiogo        360  ............................................................  419
matKR3 Sour           360  ..............................................C.............  419
matKR5 Tunisia        360  ............................................................  419
matKR7 Venezuela      360  ............................................................  419
matKR8 Hadramout      360  ............................................................  419
matKR9 Lawdar         360  ............................................................  419

matKR2 Egypt          420  ATCTATATAAATTCATTATCTAAGCATTCTCTCAACTTTTTGGGCTATCTTTCAAATGTA  479
matKR6 UAE            420  ............................................................  479
matKR1 Kadiogo        420  ............................................................  479
matKR3 Sour           420  ..............................................G.............  479
matKR5 Tunisia        420  ............................................................  479
matKR7 Venezuela      420  ............................................................  479
matKR8 Hadramout      420  ............................................................  479
matKR9 Lawdar         420  ............................................................  479

matKR2 Egypt          480  CAATTTAATCCTTCGTTGGTACGGAGTCAAATGAAAGAAAAT  521
matKR6 UAE            480  ................................T.........  521
matKR1 Kadiogo        480  ................................T.........  521
matKR3 Sour           480  ................................T.........  521
matKR5 Tunisia        480  ................................T.........  521
matKR7 Venezuela      480  ................................T.........  521
matKR8 Hadramout      480  ................................T.........  521
matKR9 Lawdar         480  ................................T.........  521
```

Figure 3. Part aligned sequence of the *matK* barcode region of eight accessions of *R. communis*. The dots indicate that the base at that position in the specified sequence is the same as the base in the sequence written at the top of the compilation.

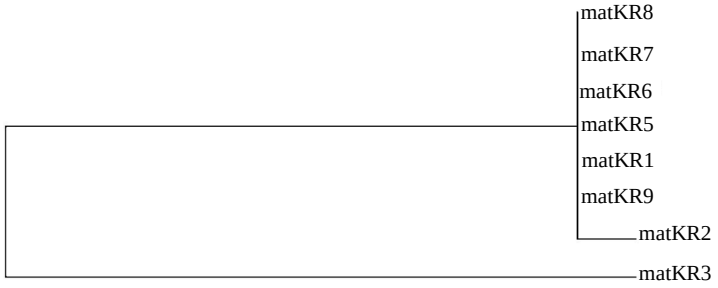

Figure 4. Maximum likelihood tree constructed by partial sequence of *matK* gene from eight *R. communis accession.*

usually sufficient [15,26]. The *matK* region varied sufficiently to distinguish "*Sanqi*" (*Panax notoginseng*; Araliaceae) from different geographical origins [27], but it failed to differentiate among *Sanqi* cultivars [28]. The partial matK sequence of 7 accessions (R1, R5, R6, R7, R8, and R9) shows only one nucleotide substations at

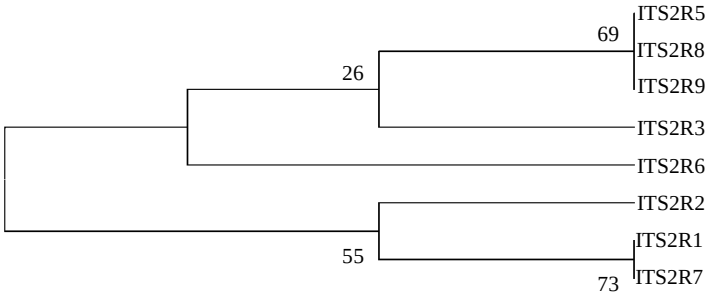

Figure 5. Maximum likelihood tree constructed ITS2 sequence from eight *R. communis accessions.*

one position compared with accession R2 from Egypt. However, R3 accession from Lebanon had 16 base substitutions. In this study accessions from Yemen, Kadiogo, Venezuela, United Arab Emirates and Tunisia had the same *matK* sequence which might be ascribed to the same ancestor and different Environment. In the present study, nrITS2 sequence was found to correlate with geographical distributions of the samples which matK gene sequence was conserved than the ITS2.

5. Conclusion

Based on our own findings, we propose that ITS2 be used as the desired barcode to study geographical distributions of Euphorbiaceae species.

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