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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(2): 1474-1484. **OPEN ACCESS**

Authentication of *Cordia dentata* Poir. Growing in Egypt using ISSR and DNA barcoding

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Cordia dentate was introduced to Egypt as ornamental and timber trees in the beginnings of the 19th Century. Urbanization is responsible for disappearance of many plant species including *C. dentata* that are represented with only two trees exhibiting different morphological characteristics. The present study aimed to authenticate these trees using rbcl- and matk-based DNA barcoding as well as ISSR markers. Results reflected that matk and rbcl sequences for both trees were 100% identical and showed 100% similarities with corresponding sequences recorded for *C. dentate* in BOLD System and Gene Bank. Nine ISSR primers, out of ten, reflected polymorphism between the two trees. Thus it is recommended to use DNA barcoding in species identification then ISSR for further intraspecific resolution.

Keywords: *Cordia dentate*, biodiversity, rbcl, matk, ISSR

INTRODUCTION

Biodiversity is a general term used to describe the sum of all life's varieties in a defined location or even across the whole planet. It occurs at ecosystem, species and genetic levels (Glowka et al., 1994). Plant biodiversity is a major source for food and drug and constitutes a natural reservoir for genetic raw material essential for breeding programs of many important crops (Rao, 2004). However, the human activities associated with over-usage of plant resources in parallel with the over-production of pollutants exaggerate the rate of plant extinction reaching one species per day (Hilton-Taylor, 2000).

The Egyptian territories host 2088 species belonging to 742 genera of 120 families (Khedr et al., 2002). *Cordia* L. (family *Cordiaceae)* is a large pantropical genus including about 300 species of trees and shrubs, distributed in Africa, South Asia and tropical America (Mabberley, 2008). In the Egyptian flora, *Cordia* was monospecific genus represented by *C. sinensis* (El Hadidy and Boulos, 2009).

Along with another seven *Cordia* species, *C. dentata* was introduced to Egyptian gardens in the beginnings of the 19th Century as ornamental and timber trees (Ascherson and Schweinfurth, 1887; Delchevalerie, 1899; Bircher and Bircher, 2000; Diwan et al., 2004; Hamdy, 2010). *C. dentate* is also rich in valuable compounds (eg: Rosmarinic acid, Quercetin, 3-o-rutinoside and Rutin) important for treatment of many human diseases (Thirupathi et al., 2008; Hossan et al., 2014; Wang et al., 2015; Ganeshpurkar and Saluja, 2017). The growing urban activities have demolished many gardens and are responsible for disappearance of many plant species. Consequently, *C. dentata* in Egypt is represented with only two individuals growing in zoological garden; the first is typical *C. dentata* while the second is *C. dentata* form (Amer et al., 2016).

Identification and characterization of endangered plant species is a prerequisite to maintain biodiversity (Bapat et al., 2012).

Traditional approaches employing morphological features require taxonomic expertise and usually suffer from subjective biases (Costion et al., 2011). Chromatographic profiles also have some limitations being affected with plant age, tissue source, physiological conditions and environmental factors (Joshi et al. 2004; Zhang et al., 2007). On the other hand, DNA based markers can be used to characterize biodiversity without fear from the previous sources of error (Bafeel et al., 2012).

An arsenal of non-sequence based molecular markers are available for biodiversity documentation, the most common of which are fragment length polymorphisms (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), inter simple sequence repeats (ISSR) and randomly amplified polymorphic DNA (RAPD) (Ganie et al., 2015 for review). RAPD and ISSR are free of many limitations facing other markers; they are time, labor and cost effective and do not necessitate prior information about sequences of the target organism genome (Muzila et al., 2014). However, the longer primers used in ISSR, compared with RAPD ones, make it more specific with higher stringent amplifications (Wolfe et al., 1998). In addition, the abundance of target sequences with high evolution rate for ISSR primers helps in revealing more polymorphic loci, compared with RAPD (Ansari et al*.,* 2012).

DNA barcoding provides another arsenal of molecular markers that are now regularly used for biodiversity inventories (Costion et al., 2011; de Vere et al., 2012). It can be defined as employing of short uniform nuclear or organelle DNA sequences (400-800bp) for the identification of different taxa (Ganie et al., 2015). The slow substitution rates and intramolecular recombination exhibited by plant mitochondrial DNA (Mower et al., 2007) in addition to the numerous recorded cases of incomplete concerted evolution of the internal transcribed spacers (ITS) in plants (Chase et al., 2005; Kress et al., 2005) put plastid sequences as front runners in plant DNA barcoding (Hollingsworth, 2008).

Considering sequence quality, recoverability and levels of species discrimination, the Consortium for the Barcode of Life (CBOL) plant working group recommended employing *rbcl* and/or *matK* in barcoding of land plants (CBOL Plant Working Group, 2009). *rbcl* is considered as universal barcode due to its high amplification success rate but it has low discriminatory potential. Conversely, *matk* gives better resolution but with some amplification concerns (Laiou et al. 2013). It recommended to use a combination of these two markers for better results (Ganie et al., 2015).

The aim of this study is to authenticate the *C. dentata* trees growing in Egypt and characterize the differences between typical *C. dentata* and *C. dentata* form using ISSR markers along with DNA barcoding using *rbcl* and *matk* sequences.

MATERIALS AND METHODS

Total genomic DNA was extracted from about 20 mg liquid nitrogen powdered leaf tissues collected from each of typical *C. dentata* and *C. dentata* form trees with aid of Qiagen DNeasy kit (Valencia, California, USA), following the manufacturer's protocols.

PCR amplifications were carried out using 17-19 base primers (Table 1) selected based on their ability to produce clear reproducible banding pattern. The reaction mixture comprised of 25 µl containing one unit *Taq* polymerase (Promega, WI, USA), 30 pmol of primer, 0.5 µl dNTPs (10 mM), 30 ng template DNA and 1.5 µl $MgCl_2$ (25 mM). The amplification protocol was initial denaturation of 2 min at 94°C; 40 cycles of 30 Sec denaturation at 94°C, 30 Sec annealing at 50°C and 2 min extension at 72°C; and final elongation step at 72°C for 7 min. PCR products were resolved in 1.5% (m/v) agarose gel and visualized under UV light. Band size was determined using Gel-Doc XR (Bio-Rad) based on 100 bp DNA ladder. Only bands appeared in three PCR amplifications were scored.

For *matk*, and *rbcl* PCR amplifications were conducted following CBOL Plant Working Group (2009) employing specific primers (Table 1) in a total volume of 50 µl containing about 50 ng genomic DNA, 1 µl of each primer and 25 µl PCR Master Mix (Bioline). The amplification protocol for *rbcl* was 95℃ for 2 min followed by 34 cycles of 94℃ for 1 min, 55℃ for 30 sec and 72℃ for 1 min, then final extension for 7 min at 72℃. *matk* protocol started with 5 min at 94 ℃ then 26 cycle of 94℃ for 1 min, 48℃ for 30 sec and 72℃ for 1 min. The final extension step lasted for 7 min at 72 ℃.

Amplicons for *rbcl* and *matk* were subjected to purification step employing the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) before sequencing using Big-dye terminator chemistry in 3130xl Genetic Analyzer (Life Technologies, California, USA) according to the standard manufacturer's protocol.

Table 1. Primer sequences

Maker	Primer	Sequence
ISSR	ISSR- 1	5'-AGAGAGAGAGAGAGAGYC-3'
	ISSR- 2	5'-AGAGAGAGAGAGAGAGYG-3'
	ISSR- 3	5'-ACACACACACACACACYT-3'
	ISSR- 4	5'-ACACACACACACACACYG-3'
	ISSR- 5	5'-GTGTGTGTGTGTGTGTYG-3'
	ISSR- 6	5'-CGCGATAGATAGATAGATA-3'
	ISSR- 8	5'-AGACAGACAGACAGACGC-3'
	ISSR- 9	5'-GATAGATAGATAGATAGC-3'
	ISSR- 10	5'-GACAGACAGACAGACAAT-3'
	ISSR- 11	5'-ACACACACACACACACYA-3'
rbcl	1f	5'-ATGTCACCACAAACAGAAAC-3'
	724r	5'-TCGCATGTACCTGCAGTAGC-3'
matk	390F	5'-CGATCTATTCATTCAATATTTC-3'
	1326R	5'-TCTAGCACACGAAAGTCGAAGT-3'

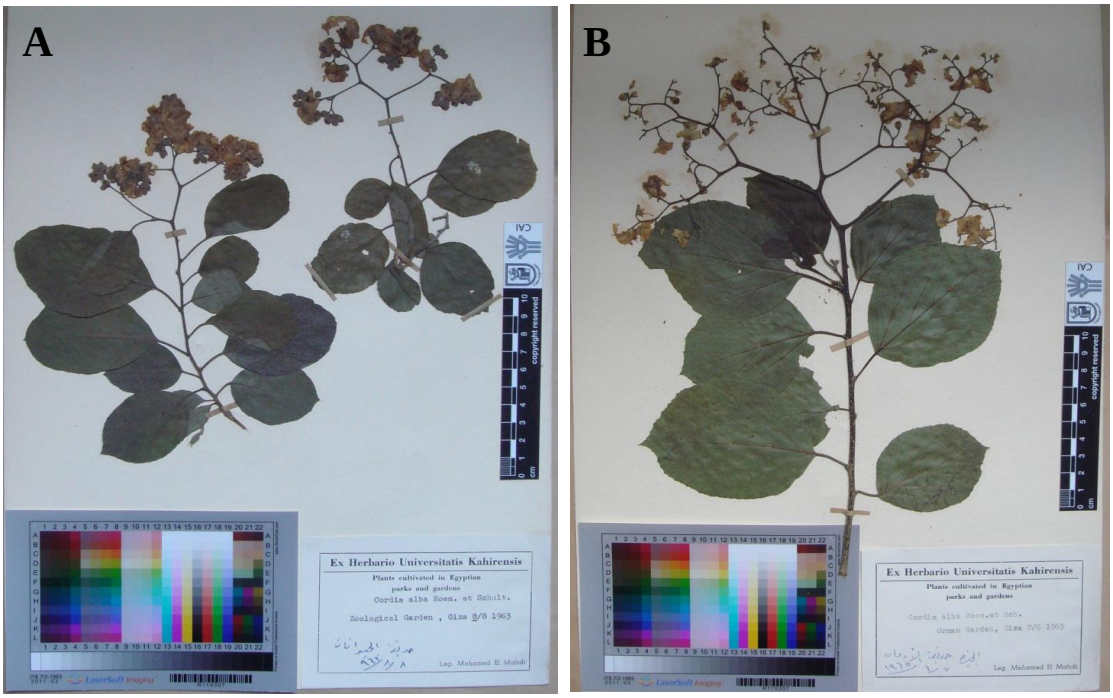


Figure 1. Twig with leaves and inflorescence of *Cordia dentata*, taken from Cairo University Herbarium (CAI), A: Typical, Zoological Garden (1965); B: Form, Orman Garden (1963).

Using Codon Code Aligner software, v. 7.1.2., forward and reverse sequences for each locus were assembled and contig sequences were used in next steps. For each locus, sequences were aligned using multiple sequence alignment (multalin) (Corpet, 1988, http://multalin.toulouse.inra.fr/multalin) for comparison. All sequences were identified using BOLD (Barcode of Life Database) System (www.boldsystems.org) and blasted using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against corresponding sequences deposited in Gene Bank.

RESULTS AND DISCUSSION

Morphological characters (Figure 1)

A small to medium-sized, semi-deciduous tree, up to 12 m high, much branched, with fibrous light-brown bark; bole seldom straight, and without

buttresses. Young vegetative parts puberulent to densely pubescent with white indumentum. Leaves alternate, broadest part at the middle or above; blade ovate, suborbicular to obovate rarely elliptical, light green in color, 3-7 cm long, 2-5.5 cm in diameter, 3-7 x 2-5.5 cm, less than one and half times as long as broad; apex obtuse to rounded; base broadly obtuse or rounded; margin sub entire to dentate; upper surface scabrous with long white hairs arise from a cystolith, lower surface pubescent, tawny hairs intermingled with ferruginous indumentum especially along the midrib and veins; midrib prominent below and flat above; secondary veins 3-5 pairs, the basal pair reaching the margin at or below the middle of the lamina; petiole terete to slightly grooved, 1-3 cm long, shorter than the leaf blade, a ppressed-pubescent with ferruginous indumentum.

Inflorescence lax, terminal cymose, less divaricately branched, 10-30 flowered; peduncle 1.5-3.5 cm long, flower buds 2-3.5 cm long, ovate, sessile; calyx campanulate, 10(-12) ribbed, sparsely strigose, 2-3.5 mm long, circumcissile and open somewhat unevenly.

Corolla yellow, 8-12 mm long, obovate with emarginated apex; stamens 5(-6), 5-9 mm long; filaments glabrous, 5-7 mm long, hairy at base anther oblong, 1-2 mm long. Gynoecium -5.5 mm long; ovary ovoid, 1-2 mm long; style included 2.5-6 mm long; stigma lobes clavate, 0.5 mm long. Fruit drupe, translucent white borne on a cup-shaped calyx, ovoid with apiculate apex, 9-13 mm long, 6-8 mm in diameter; pyrene ellipsoid, 811 mm long and 4-5 in diameter.

Morphological description of the closely allied form revealed notable variation from the typical *C. dentata* distinguished by its lamina elliptic rarely ovate, dark green in colour, 4-11 x 2.5-7 cm, 1-2 times as long as broad; apex acute to apiculate; base broadly cuneate; margin dentate rarely subentire, the basal pair reaching the margin above the middle of the lamina. In addition to, the inflorescence is dense, much divaricately branched; 30-60 flowered and the corolla is white with yellow throat.

Both trees differ in many features of external morphology including, leaf shape, size, L/W ratio, colour, apex, base and margin. This result is aligned with the observation of Johnston (1948) in some *Cordia* species among which *C. toqueve* Abl. and *C. macrophylla*, Critchfield (1960) in *Populus trichocarpa*, Smith (1967) in *Liquidambar styraciflua* L. and, Deschamp and Cooke (1985) in *Callitriche heterophylla*.

As well, Al-Turki and Thomas (2010) and Johnston (1948) stated that floral dimorphism was observed in *Moltkiopsis* I.M.Johnst. and *Cordia diversifolia*, respectively. This is in line with our study that reflects variation in inflorescence and flower colour. So the results confirm the presence of two different forms in *Cordia dentata*.

Molecular analyses

The potential of ISSR analysis was used to assess genetic variation between typical *C. dentata* and *C. dentata* form. A total of 73 bands were generated using 10 primers with an average of 7.3 band per primer (Figure 2 and Table 2). Each primer produced a unique banding pattern of 4 (using ISSR 4) to 12 (using ISSR 1) amplicons. Both trees showed the same amplification products using ISSR 2. Otherwise, each primer revealed a unique characteristic banding pattern for each tree with a total of 28 polymorphic bands (38.4% polymorphism). ISSR 8 exhibited the highest discrimination between typical *C. dentata* and *C. dentata* form. Among 6 bands it produced, 5 (83.33%) were polymorphic suggesting that this primer amplifies genetically unstable hypervariable regions (Hollingsworth et al., 1997). On the other hand, ISSR 9 had the lowest discriminatory potential where it revealed only 20% polymorphism between both trees.

In the available literature, only Brito et al., (2016) studied intraspecific genetic variation in *Cordia* employing ISSR analysis. Compared with our results, the authors recorded higher intraspecific variation designated with about 93% polymorphism using 14 ISSR primers in *C. verbenacea*. High % polymorphism was also recorded in apricot (Kumar et al., 2009), *Achilleatenuifolia* (Rahimmalek, 2012), *Daemonorops draco* (Asra et al., 2014), *Croton tetradenius* (Almeida-Pereira et al., 2017) and strawberry (Kaleybar et al., 2018). However, the smaller intraspecific genetic variation recorded in this study can be explained by presence in small geographic range (Liu et al., 2013).

Bidirectional sequences for both *rbcl* and *matk* for typical *C. dentata* and *C. dentata* form tress were obtained. In both trees, *matk* sequence length was 641 bp while *rbcl* was 501 bp. Identifications for both sequences of both tress showed 100% similarity to the corresponding *C. dentata* sequences deposited in BOLD system and Gene Bank data bases (Figures 3 and 4) reflecting validity of DNA barcoding using such loci at species level.

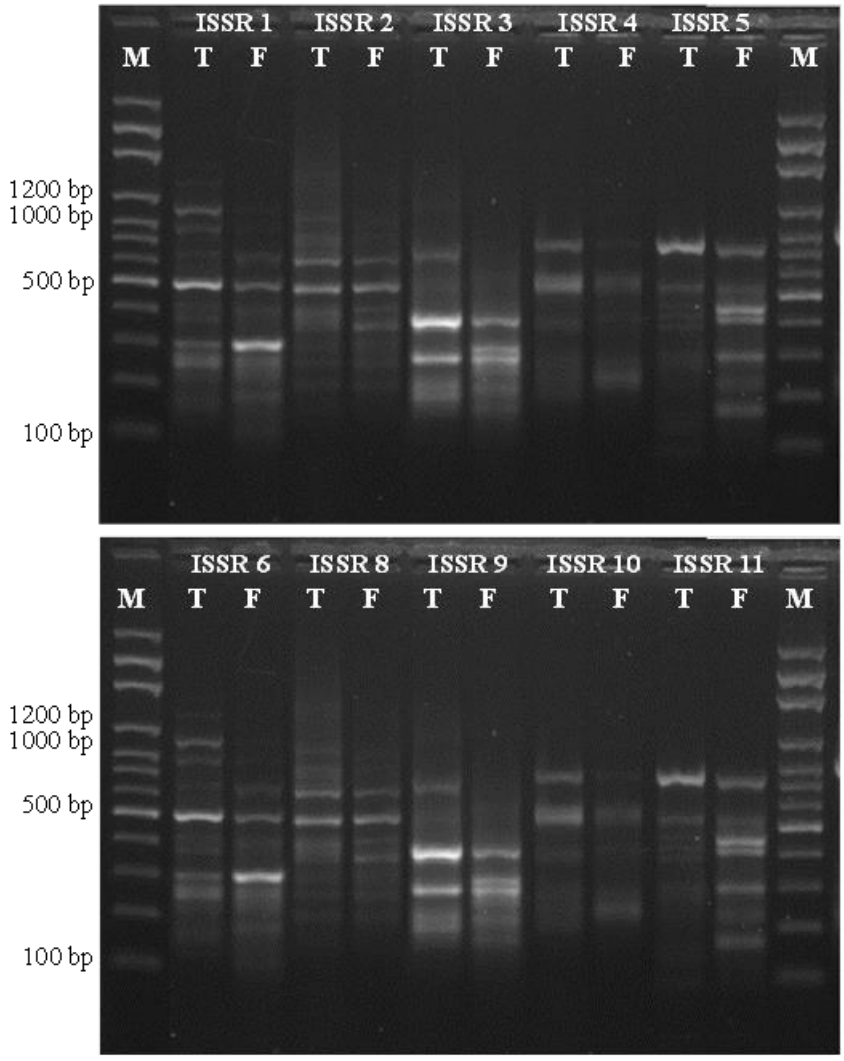
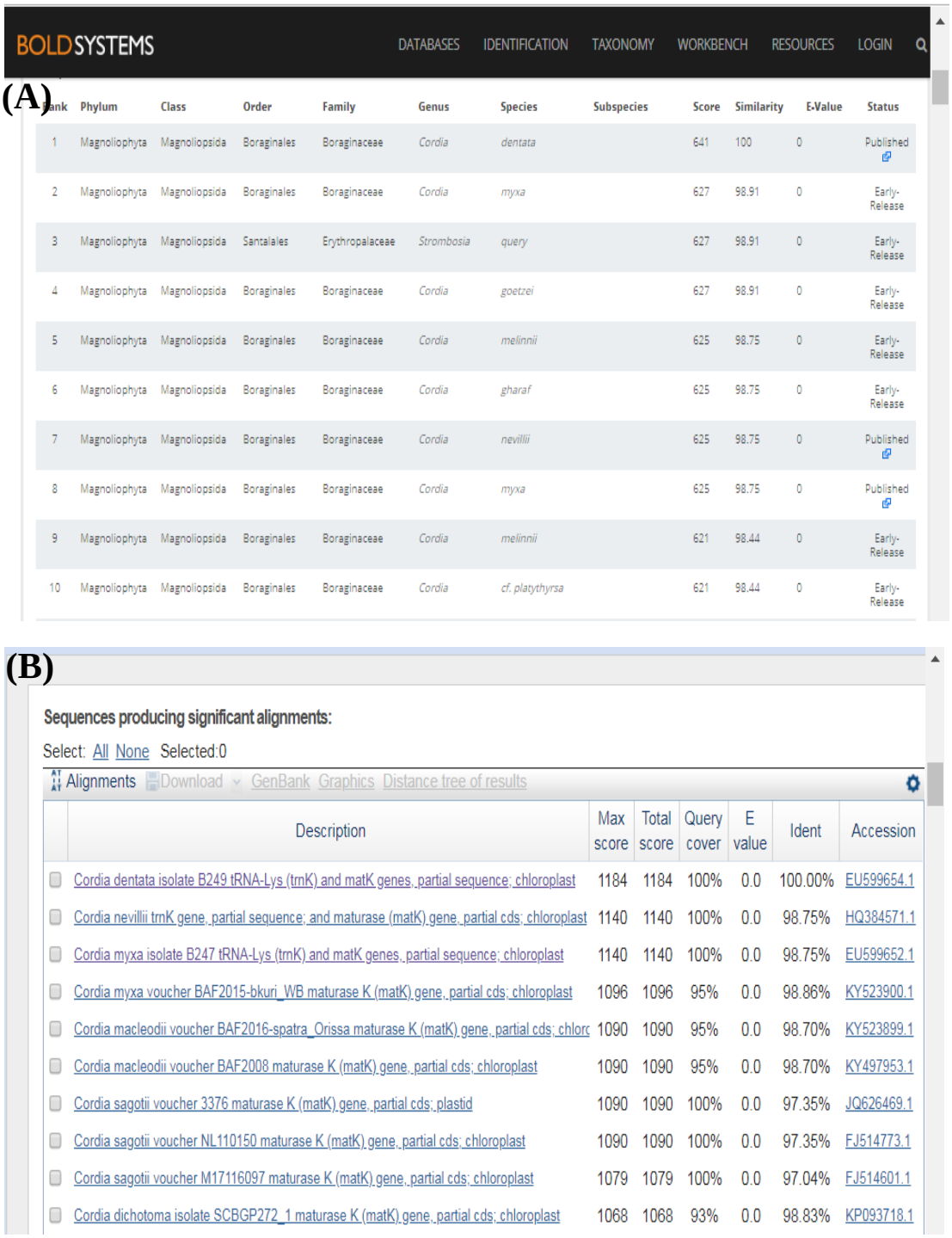


Figure 2. ISSR banding patterns of typical *Cordia dentata* (T) and *C. dentata* form (F) using 10 primers.

Table 2. ISSR analyses of typical *Cordia dentata* and C. *dentata* form.

Primer	Total No. of bands	Monomorphic bands	Polymorphic bands	% of polymorphim
ISSR- 1	12	9	3	25.00
ISSR- 2	9	9	0	0.00
ISSR- 3	7	4	3	42.86
ISSR- 4	4	3	1	25.00
ISSR- 5	8	6	2	25.00
ISSR- 6	7	3	4	57.14
ISSR- 8	6	1	5	83.33
ISSR- 9	5	4	1	20.00
ISSR- 10	9	2	7	77.78
ISSR- 11	6	4	2	33.33
Total	73	45	28	38.36



(A)

BOLD SYSTEMS — DATABASES | IDENTIFICATION | TAXONOMY | WORKBENCH | RESOURCES | LOGIN

Rank	Phylum	Class	Order	Family	Genus	Species	Subspecies	Score	Similarity	E-Value	Status
1	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dentata*		641	100	0	Published
2	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*myxa*		627	98.91	0	Early-Release
3	Magnoliophyta	Magnoliopsida	Santalales	Erythropalaceae	*Strombosia*	*query*		627	98.91	0	Early-Release
4	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*goetzei*		627	98.91	0	Early-Release
5	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*melinnii*		625	98.75	0	Early-Release
6	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*gharaf*		625	98.75	0	Early-Release
7	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*nevillii*		625	98.75	0	Published
8	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*myxa*		625	98.75	0	Published
9	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*melinnii*		621	98.44	0	Early-Release
10	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*cf. platythyrsa*		621	98.44	0	Early-Release

(B)

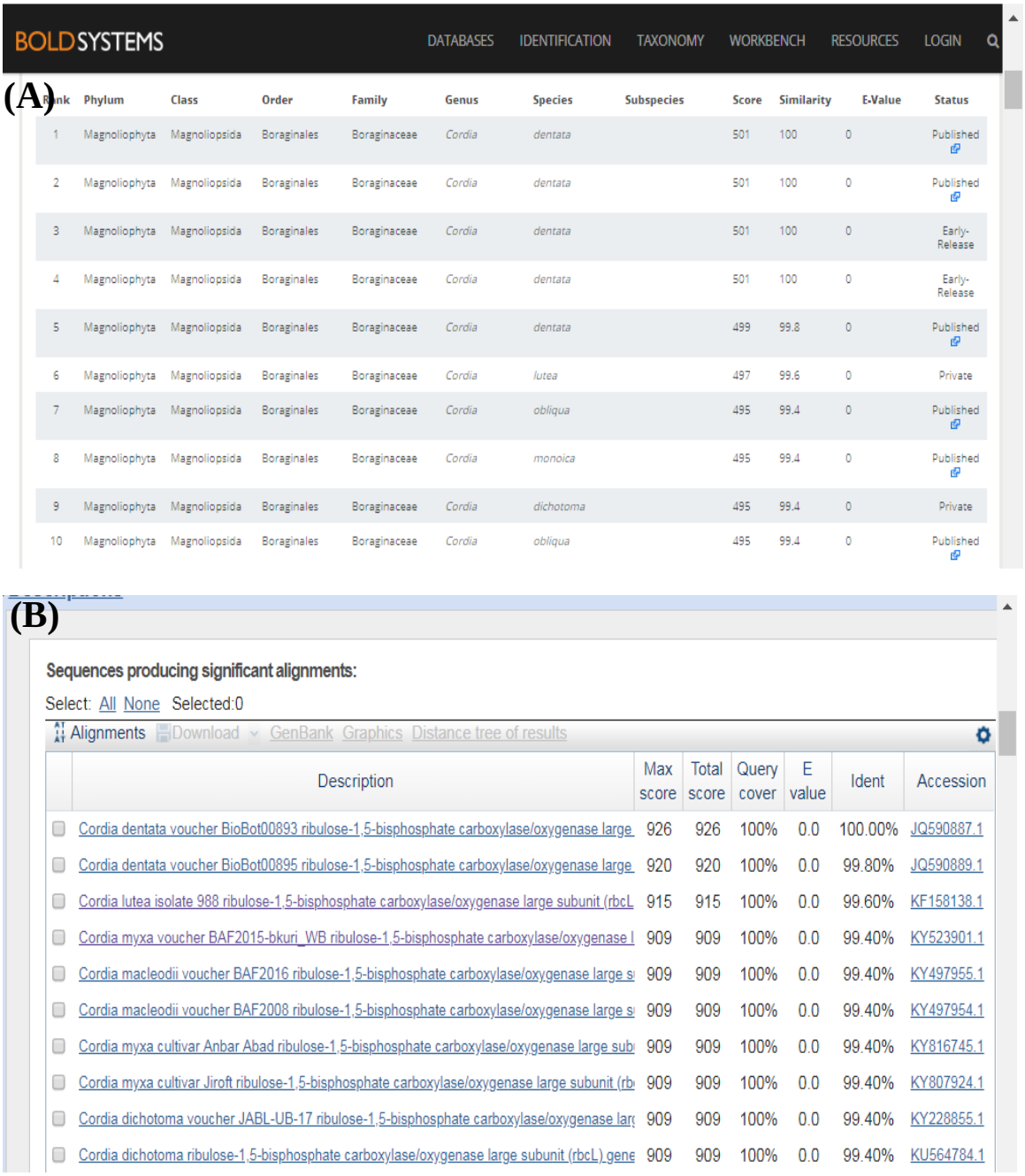
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Cordia dentata isolate B249 tRNA-Lys (trnK) and matK genes, partial sequence; chloroplast	1184	1184	100%	0.0	100.00%	EU599654.1
Cordia nevillii trnK gene, partial sequence; and maturase (matK) gene, partial cds; chloroplast	1140	1140	100%	0.0	98.75%	HQ384571.1
Cordia myxa isolate B247 tRNA-Lys (trnK) and matK genes, partial sequence; chloroplast	1140	1140	100%	0.0	98.75%	EU599652.1
Cordia myxa voucher BAF2015-bkuri_WB maturase K (matK) gene, partial cds; chloroplast	1096	1096	95%	0.0	98.86%	KY523900.1
Cordia macleodii voucher BAF2016-spatra_Orissa maturase K (matK) gene, partial cds; chlorc	1090	1090	95%	0.0	98.70%	KY523899.1
Cordia macleodii voucher BAF2008 maturase K (matK) gene, partial cds; chloroplast	1090	1090	95%	0.0	98.70%	KY497953.1
Cordia sagotii voucher 3376 maturase K (matK) gene, partial cds; plastid	1090	1090	100%	0.0	97.35%	JQ626469.1
Cordia sagotii voucher NL110150 maturase K (matK) gene, partial cds; chloroplast	1090	1090	100%	0.0	97.35%	FJ514773.1
Cordia sagotii voucher M17116097 maturase K (matK) gene, partial cds; chloroplast	1079	1079	100%	0.0	97.04%	FJ514601.1
Cordia dichotoma isolate SCBGP272_1 maturase K (matK) gene, partial cds; chloroplast	1068	1068	93%	0.0	98.83%	KP093718.1

Figure 3. *matk*-identification of typical *Cordia dentata and C. dentata* form using BOLD System (A) and Gene Bank data bases (B).



Rank	Phylum	Class	Order	Family	Genus	Species	Subspecies	Score	Similarity	E-Value	Status
1	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dentata*		501	100	0	Published
2	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dentata*		501	100	0	Published
3	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dentata*		501	100	0	Early-Release
4	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dentata*		501	100	0	Early-Release
5	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dentata*		499	99.8	0	Published
6	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*lutea*		497	99.6	0	Private
7	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*obliqua*		495	99.4	0	Published
8	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*monoica*		495	99.4	0	Published
9	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*dichotoma*		495	99.4	0	Private
10	Magnoliophyta	Magnoliopsida	Boraginales	Boraginaceae	*Cordia*	*obliqua*		495	99.4	0	Published

Description	Max score	Total score	Query cover	E value	Ident	Accession
Cordia dentata voucher BioBot00893 ribulose-1,5-bisphosphate carboxylase/oxygenase large	926	926	100%	0.0	100.00%	JQ590887.1
Cordia dentata voucher BioBot00895 ribulose-1,5-bisphosphate carboxylase/oxygenase large	920	920	100%	0.0	99.80%	JQ590889.1
Cordia lutea isolate 988 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL	915	915	100%	0.0	99.60%	KF158138.1
Cordia myxa voucher BAF2015-bkuri_WB ribulose-1,5-bisphosphate carboxylase/oxygenase l	909	909	100%	0.0	99.40%	KY523901.1
Cordia macleodii voucher BAF2016 ribulose-1,5-bisphosphate carboxylase/oxygenase large s	909	909	100%	0.0	99.40%	KY497955.1
Cordia macleodii voucher BAF2008 ribulose-1,5-bisphosphate carboxylase/oxygenase large s	909	909	100%	0.0	99.40%	KY497954.1
Cordia myxa cultivar Anbar Abad ribulose-1,5-bisphosphate carboxylase/oxygenase large sub	909	909	100%	0.0	99.40%	KY816745.1
Cordia myxa cultivar Jiroft ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rb	909	909	100%	0.0	99.40%	KY807924.1
Cordia dichotoma voucher JABL-UB-17 ribulose-1,5-bisphosphate carboxylase/oxygenase lar	909	909	100%	0.0	99.40%	KY228855.1
Cordia dichotoma ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene	909	909	100%	0.0	99.40%	KU564784.1

Figure 4. *rbcl*-identification of typical *Cordia dentata and C. dentata* form using BOLD System (A) and Gene Bank data bases (B).

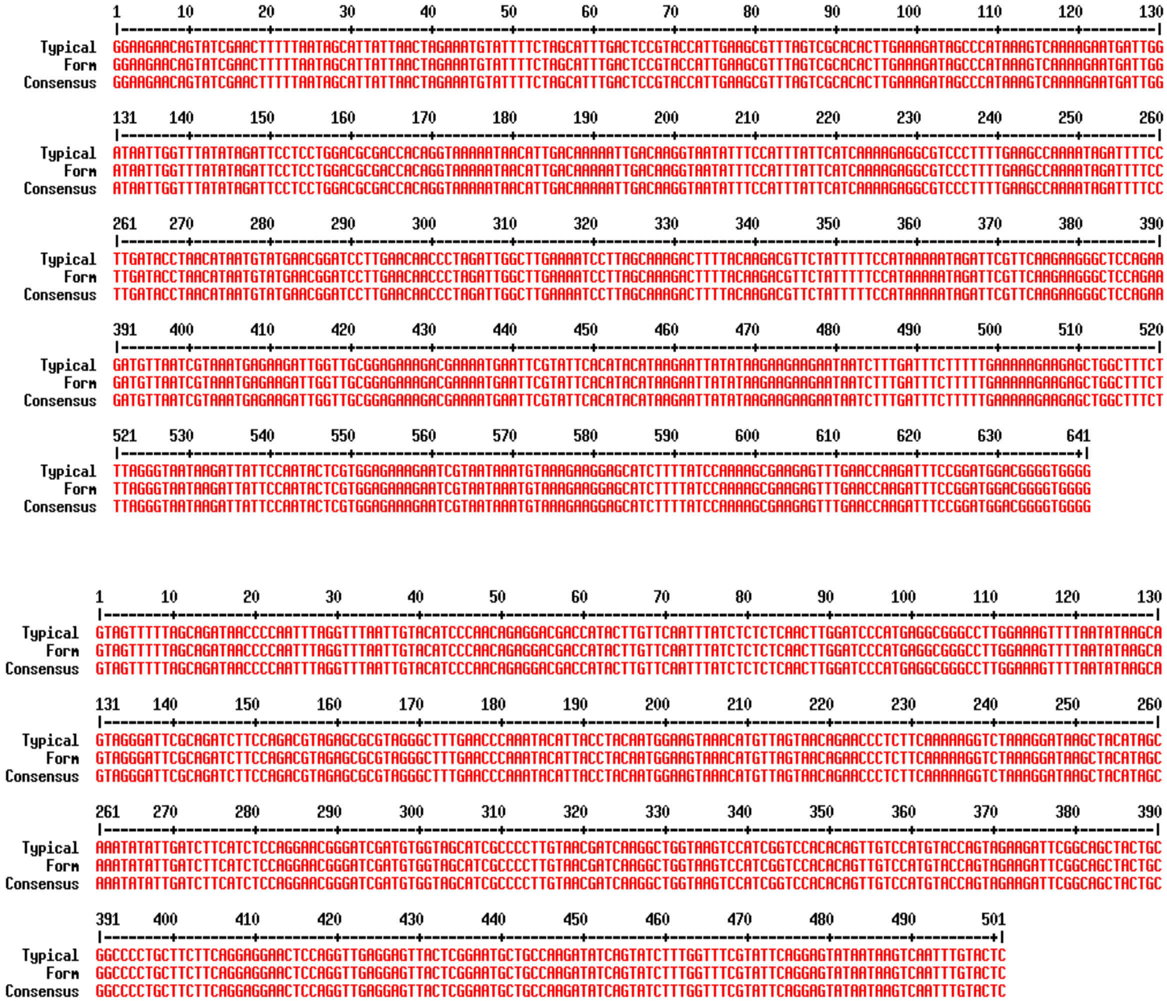


Figure 5. Multalin-based sequence alignment for *matk* (A) and *rbcl* (B) sequences of typical *Cordia dentata and C. dentata* form.

However, multalin sequence alignments for each of *rbcl* and *matk* sequences showed that both typical *C. dentata* and *C. dentata* form tress are 100% identical (Figure 5) suggesting recent divergence of *C. dentata* form (Kerr et al., 2007) or divergence from a strong common genetic basis (Baraket et al., 2011).

Similar failure of DNA barcoding in characterization of intraspecific variation was recorded in *Cordia macleodii* (Deb et al., 2018) and several species including *Panax notoginseng* **(**Zhang et al., 2006), *Phaseolus* species (Nicolè et al., 2011), *Sansevieria trifasciata* (Tallei et al., 2016) and *Codia eumvariegatum* (Nio et al., 2018). On the other hand, DNA barcoding was used successfully to monitor intraspecific variation in *Phoenix dactylifera* (Enan and Ahmed, 2014) and *Ficu scarica* (Castro et al., 2015). These contradictory literatures suggest the influence of species genotype on efficiency of DNA barcoding in resolving intraspecific variation.

CONCLUSION

In conclusion, matk- and rbcl-based DNA barcoding are efficient tools for identification of *C. dentata*. However, both sequences are not variable enough to resolve different forms of such species even in presence of morphological differences. ISSR is a potent molecular marker able to produce sufficient polymorphism to fine resolve intraspecific genetic variation. Thus it is recommended to use both molecular approaches

to authenticate *C. dentata* in Egypt, DNA barcoding for identification at species level and ISSR for characterization of intraspecific variations.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

No acknowledgment for anybody.

AUTHOR CONTRIBUTIONS

Rim Hamdy collected fresh materials from the studied trees, examined using stereomicroscope and identified the specimens using compared material kept in CAI Herbarium and wrote morphological part. Ahmed Sayed Fouad and Rehab Mahmoud Hafez shared equally molecular analysis including DNA extraction, primers selection, PCR and analysis of DNA sequences and prepared the related parts in manuscript.

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