# Ribulose-1, 5-biphosphate carboxylase (rbcL) gene sequence and random amplification of polymorphic DNA (RAPD) profile of regionally endangered tree species *Coptosperma graveolens* subsp. *arabicum* (S. Moore) Degreef

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## Abstract

*Coptosperma graveolens* subsp. *arabicum* (Rubiaceae) is a shrub or small tree and currently endangered in Saudi Arabia. In this study, ribulose-1, 5-biphosphate carboxylase (rbcL) gene sequence and random amplification of polymorphic DNA (RAPD) profile of *C. graveolens* were determined. Our study showed that this species is phylogenetically related with *Tarenna supra axilaris*, *Coptosperma* sp. and *Paracephaelis* sp. group on the basis of rbcL gene sequences. The studied specimen and related taxa inferred from partial rbcL gene sequence demonstrated a distinct lineage; therefore, could distinguish the species as *C. graveolens* unequivocally. The specimen (6R1) demonstrated the highest level of sequence similarities (99.5%) with three close species in the group. RAPD profile of *C. graveolens* generated amplified products ranging from 218 to 1338 bp. A total of 18 bands were observed for plant species using 5 commercially available RAPD primers (P). The number of major bands for the plant species for a single primer ranged from 3 to 5. The maximum number of major bands was observed for the P1 (5 bands), 4 bands for P4 and the equal number of bands (3) for P2, P3 and P5. The determined rbcL gene sequence and RAPD profile of the endangered plant species *C. graveolens* will improve the identification process with morphology based taxonomic methods.

**Keywords:** *Coptosperma graveolens* subsp. *arabicum*; RAPD; rbcL.
**Abbreviations:** BLAST - Basic Local Alignment Search Tool; BOLD - The Barcode of Life Data Systems; CBOL - The Consortium for the Barcode of Life; *C. graveolens* - *Coptosperma graveolens*; RAPD - Random Amplification of Polymorphic DNA; rbcL - Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit.

## Introduction

*Coptosperma graveolens* (S. Moore, Bremek) Degreef is a shrub or small tree, usually 2–9 m in height, found in bush land, drier forest (margins/riverine) thickets and shrubby grassland. This species is distributed in tropical Arabia and Africa (Fischera et al., 2010). This species belongs to the tribe Pavetteae under the family Rubiaceae. *C. graveolens* has been used as traditional medicine (Barnett, 2000). Its roots are reported to be used for the cure of pneumonia (Mwangi, 2010). Bark, leaves and roots (prepared by pounding and administered orally) are used in sexual impotence and erectile dysfunction in western Uganda (Mugisha and Origa, 2005). Bark or roots are also used in treating various HIV/AIDS related opportunistic infection in Bukoba, rural district of Tanzania (Kisangau et al., 2007). This less studied species is endangered in Saudi Arabia (Collenette, 1998; Collenette, 1999). We are not aware of literatures on molecular characterization of *C. graveolens* except early description about morphological and

geographical distribution of this species (Diane and Bridson, 1979; De-Block et al., 2001; Degreef et al., 2001; Fischera et al., 2010). Plastid rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit ) is the most commonly sequenced gene for phylogenetic studies of plants (Schuettpelz et al., 2006), in which the success rate of PCR and sequencing is higher compared with the other selected plant characterization genes such as matK (maturase K) (CBOL Plant Working Group, 2009; Parveen et al., 2012). Chloroplast rbcL gene sequences representing from 90 taxa of 12 genera and 10 angiospermic plant families (dicot and monocots) unambiguously resolved the relationships, as well as provided a good indication of major supra-generic groupings among the selected angiospermic plant families (Reddy, 2009). RAPD (random amplified polymorphic DNA) provides an easy and rapid method and does not need any DNA probe or sequence information for primer design. RAPD has been used previously for estimation of genetic diversity in various endangered plant species (Wang et al.,

2005; Zheng et al., 2008). Thus, widely used rbcL gene sequence and RAPD markers may help the conservation and sustainable maintenance of this plant species. In this study, we attempted to characterize this regionally endangered plant species by rbcL gene sequence and RAPD fingerprinting.

## Results and Discussion

### *Taxonomy*

Morphological description and geographic distribution of the genus *Coptosperma* is well documented by Degreef et al. (2001). Genus *Coptosperma* currently has 11 species and 2 subspecies of *C. graveolens* (i.e. *C. graveolens* subsp. *graveolens* var. *graveolens*; *C. graveolens* subsp. *graveolens* var. *impolitum*; *C. graveolens* subsp. *arabicum*), of which *C. graveolens* subsp. *arabicum* is the only species, found in the Arabian Peninsula (Collenette, 1999; Chaudhary, 2000). *Coptosperma graveolens* (S. Moore) Degreef [Synonym, *Pavetta graveolens* S. Moore; *Tarenna graveolens* (S. Moore) Bremek] has all the generic characters of *Coptosperma* but lacks the ruminate endosperm (Bridson, 1979; Degreef et al., 2001). The *C. graveolens* morphologically resembles *C. neurophyllum* but differs from the latter by the presence of pubescent leaves (abaxial surface), subulate bracteoles and circular hilum cavity. Almost all of the *Coptosperma* species are characterized by terminal inflorescence. The unique species with axillary inflorescene is *C. supra-axilaris*. The *Tarenna graveolens* was transferred from *Tarenna sensu stricto* to the genus *Coptosperma* based on spherical seed, no thickenings of the exotesta cells and pollen with microreticulate exine.

The *C. graveolens* subsp. *arabicum* is characterized by puberulous leaves; calyx tube as long as the triangular lobes, ovate to triangular or rarely acuminate stipules, terminal inflorescence; subulate, pubescent bracteoles; ovules 2-3 (-7), 2-3 or 2-4; placentation impressed; hilar cavity is circular, pale and entire endosperm. The *C. graveolens* subsp. *arabicum* is particularly characterized with puberulous leaves, 2-4 ovules and circular hilar cavity (Degreef et al., 2001). Bridson (1979) described the Arabian material as subsp. *arabica* (Cufod.) Bridson with leaf-blades always narrowly elliptic, the inflorescences smaller than those of subsp. *graveolens* and calyx-lobes acute to more or less rounded (Fig. 1).

### *Phylogeny (Maximum Likelihood, Maximum Parsimony and Neighbor-Joining)*

BLAST (Basic Local Alignment Search Tool) search, genetic distance and tree-based methods have been commonly used for identification of species. In this process, sequence is assigned on the basis of its similarity to a set of reference (identified) sequences (Ross et al., 2008). We conducted BLAST and BOLD (The Barcode of Life Data Systems) database-search to determine the approximate identification and related taxa of the studied specimen. BLAST search showed 99% sequence similarities with multiple plant species (*Randia dryadum*, FJ976170; *Tarenna supra-axilaris*, AJ286711; *Coptosperma* sp., AM117218). BOLD database showed 99.5% sequence similarities with *Tarenna supra-axilaris*. We retrieved the related sequences from the GgenBank database to determine the phylogenetic position of

the studied specimen. Tree analyses were conducted using maximum likelihood, maximum parsimony and neighbor joining methods. The studied specimen was phylogenetically related with *Tarenna supra axilaris-Coptosperma* sp.-*Paracephaelis* sp. group (Fig. 2; supplementary Figs. 1 and 2). All the trees that were inferred from partial rbcL gene sequence of the studied specimen and related taxa demonstrated a distinct lineage of the studied specimen; thus, could distinguish the species of *C. graveolens* unequivocally. Absence of *C. graveolens* sequence and related genera in the database may be responsible for such tree-topology. There are very few rbcL records of plants on the current GenBank/BOLD identification system (v 2.5) (Ratnasingham and Hebert, 2007), so queries may not return an authentic match. Recent study showed that 13-fold expansion of taxon sampling resulted in improved phylogenetic assessment, despite of up to 38% missing data (Crawley and Hilu, 2012). The specimen demonstrated the highest level of sequence similarities (99.5%) with these plant species compared with other taxa. Overall mean sequence similarity among the studied plant species was very high (99.3%) (Table 1).

Assignment of an unknown specimen under valid taxa primarily depends on the availability of the sequence in the database. Molecular genetic techniques for species identification based on single-gene sequence similarity or phylogenies are rapidly gaining wide use (Ross et al., 2008) except few criticisms (Will and Rubinoff, 2004; Meyer and Paulay, 2005). One of the prime motivations for the development of genetic methods is their large-scale application to species identification. BLAST, distance and liberal tree-based methods showed equally success when all species are represented in the reference data set (Ross et al., 2008).

### *RAPD*

Sequence-based analyses sometimes fail to distinguish between species because of the significant similarity between their DNA sequences in the amplified region. In such instances, RAPD primers are able to distinguish taxa (Choo et al., 2009), because RAPD analysis includes both of the coding and non-coding regions of the genome (Vanijajiva et al., 2005). RAPD has been used in botanic forensic purposes (Mestel, 1993; Yoon, 1993). The RAPD-primers (P1, P2, P3, P4 and P5) used in this study were able to amplify the DNA obtained from the plant-specimen. The RAPD banding patterns of *C. graveolens* is illustrated in Fig. 3. The RAPD profile using the sample generated amplified products ranged from 218 to 1338 bp. A total of 18 bands were observed for the plant species using 5 primers. The number of major bands for the plant species for a single primer ranged from 3 to 5. The maximum number of major bands was observed for the primer P1 (5 bands), 4 bands for P4 and the equal number of bands for P2, P3 and P5 (3 bands, Fig. 3). Some of the reported problems with RAPD are related to reproducibility, designing appropriate primers and amplification of RAPD-PCR products. PCR conditions constitute another crucial factor for obtaining amplified products, especially for plants (Jones et al., 1997). However, if the overall temperature profiles (annealing temperature) inside the PCR tubes are identical, RAPD fragments are then likely to be reproducible (Penner et al., 1993; Skroch and Nienhuis, 1995).

**Table 1.** Estimates of rbcL gene sequence similarities (%) among the studied taxa. The highest sequence similarities with *C. graveolens* subsp. *arabicum* were typed in bold. Overall mean sequence similarity is 99.3%.

| No. | Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Tarenna neurophylla* | | | | | | | | | | | | | |
| 2 | *Tarenna buruensis* | 99.9 | | | | | | | | | | | | |
| 3 | *Tarenna supra axillaris* | 99.6 | 99.7 | | | | | | | | | | | |
| 4 | *Coptosperma* sp. | 99.6 | 99.6 | 99.6 | | | | | | | | | | |
| 5 | *Paracephaelis* sp. | 99.4 | 99.4 | 99.4 | 99.6 | | | | | | | | | |
| 6 | *Dictyandra arborescens* | 98.7 | 99.2 | 98.2 | 99.1 | 99.1 | | | | | | | | |
| 7 | *Leptactina platyphylla* | 98.6 | 99.0 | 98.2 | 98.8 | 98.9 | 99.8 | | | | | | | |
| 8 | *Amaioua guianensis* | 99.1 | 98.9 | 98.9 | 98.9 | 98.7 | 99.0 | 98.8 | | | | | | |
| 9 | *Sukunia longipes* | 98.6 | 99.1 | 98.6 | 99.0 | 99.0 | 98.3 | 98.3 | 98.6 | | | | | |
| 10 | *Randia schumanniana* | 99.2 | 99.1 | 99.1 | 99.1 | 99.1 | 98.8 | 98.6 | 98.8 | 99.2 | | | | |
| 11 | *Gardenia jasminoides* | 99.0 | 98.8 | 99.1 | 99.1 | 99.0 | 98.7 | 98.7 | 98.8 | 99.3 | 99.3 | | | |
| 12 | *Randia dryadum* | 99.0 | 99.0 | 98.8 | 99.0 | 99.0 | 98.5 | 98.3 | 98.8 | 98.8 | 99.9 | 99.3 | | |
| 13 | *Coptosperma graveolens* | 99.3 | 99.3 | **99.5** | **99.5** | **99.5** | 99.1 | 99.1 | 98.7 | 98.9 | 98.9 | 98.4 | 98.8 | |
| 14 | *Aidia* sp. | 98.9 | 98.8 | 98.9 | 98.7 | 98.7 | 98.9 | 98.8 | 98.7 | 99.1 | 98.9 | 99.0 | 98.9 | 98.4 |



**Fig 1**. **a**. Regionally endangered plant *C. graveolens* subsp. *arabicum* in the sample collected site (Al-Baha, Saudi Arabia); **b.** Flower; **c**. Leaf and fruits.

## Materials and methods

### Plant material

In this study, one specimen (6R1, Fig. 1) collected from Al-Baha, Saudi Arabia (19.8597001707, 41.3083145406) was investigated. The leaf samples were individually placed in plastic pouches and transported to the laboratory where the specimen was stored at -80ºC until processed for DNA extraction. The plant was morphologically identified at Herbarium, King Saud University (KSU), Riyadh, Saudi Arabia.

### DNA extraction

The leaf specimen was immersed in liquid nitrogen and crushed using sterile mortar and pestle to get a fine powder. DNeasy plant mini kit (Qiagen) and an automated DNA extraction instrument (QIAcube, Qiagen) were used for DNA isolation. Quality of the extracted DNA was determined using gel electrophoresis and Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Isolated plant genomic DNA was preserved at -80ºC.

### PCR

A set of primer, rbcLaF (5′ATGTCACCACAAACAG-AGACTAAAGC3′; Levin et al., 2003) and rbcLaR (5′GTAAAATCAAGTCCACCRCG3′; Kress and Erickson, 2007) was used in this study for the amplification of rbcL gene of the chloroplast. A total volume of 30 µl of PCR reaction mixture prepared as follows: 15 µl of FideliTaq PCR Master Mix (USB Corporation, Cleveland, OH), a final concentration of 200 µM of each dNTPs and 1.5 mM $MgCl_2$, 1µM of each primer (Eurofins MWG Operon, Germany), 2 µL of genomic DNA, adjusted with sterile distilled water (to 30 µl). The PCR amplification was performed with a Veriti 96 well plate thermal cycler (Applied Biosystems) as follows: 95°C for 1 min, followed by 35 cycles of 95°C for 30s, 51°C for 30s and 68°C for 1 min, followed by an final elongation step at 68°C for 5 min.
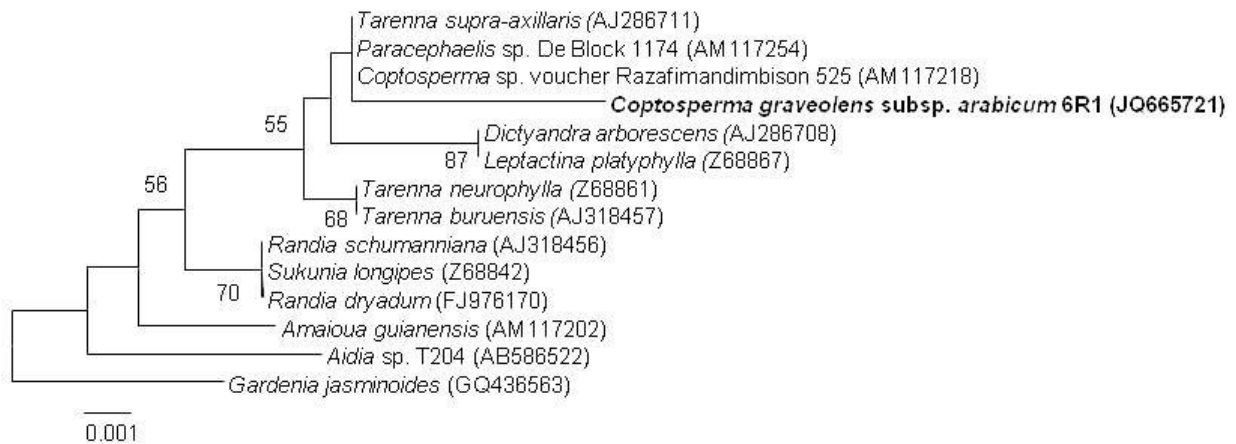
**Fig 2.** The Maximum Likelihood tree showing the relationship of *C. graveolens* subsp. *arabicum* with the related taxa. GenBank accession numbers of the corresponding taxa are written in parentheses. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown (>50%) next to the branches. The scale bar represents the branch length measurement in the number of substitutions per site.
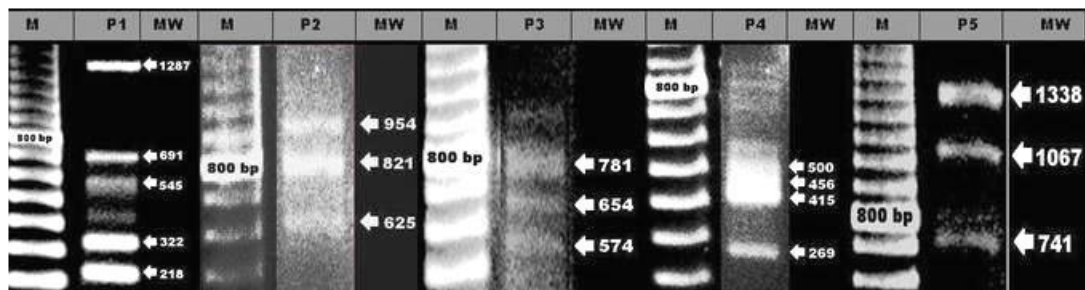


**Fig 3.** RAPD-PCR products of *C. graveolens* subsp. *arabicum* for different primers. M, 100-bp molecular weight marker; P, Ready-To-Go RAPD analysis primers (GE Healthcare, Buckinghamshire, UK); MW, Molecular Weight.

### Agarose gel electrophoresis

A long (20 × 14 cm) 1% agarose gel using 1X TAE buffer containing 0.5 μg/mL ethidium bromide was used for electrophoresis of PCR-products. Gel images were obtained using Proxima C16 Phi+ (Isogen Life Science) UV transilluminator and Opticom (version 3.2.5; OptiGo) imaging system. The amplified PCR products were determined on gel for the presence or absence of the band. The size of PCR products resulting from the primer pair were determined by using an Amersham 100-bp ladder (GE Healthcare) and the TotalLab TL100 1D software (version 2008.01).

### Sequencing

Sequences were determined directly using the dideoxynucleotide chain-termination method with a DNA-Sequencer (ABI PRISM 3130xl; Applied Biosystems/Hitachi) and BigDye Terminator (version 3.1) cycle sequencing kit (RR-100, Applied Biosystems), according to the manufacturer's instructions. Obtained rbcL gene sequence was submitted to DDBJ/EMBL/GenBank database (Accession no. JQ665721).

### Assignment of taxa

BLAST and BOLD searches were applied to the produced sequence using the available online databases. Sequences of rbcL that matched closely with the query sequences were retrieved from DDBJ/EMBL/GenBank database. The sequences were aligned using CLUSTAL X (version 1.81) (Thompson et al., 1997). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Phylogenetic trees were constructed using Maximum Likelihood (ML; Tamura and Nei, 1993), Maximum Parsimony (MP; Nei and Kumar, 2000) and Neighbor-Joining (NJ; Saitou and Nei, 1987) methods. The topologies of the phylogenetic trees were evaluated using the bootstrap re-sampling method of Felsenstein (1985) with 1000 replicates.

### RAPD-PCR analysis

Ready-To-Go RAPD analysis beads (GE Healthcare, Buckinghamshire, UK) were used for RAPD-PCR analysis. The PCR mixture of 25 μL contained a single Ready-To-Go RAPD analysis bead, 25 pmol of a single RAPD primer, 50 ng of template DNA and sterile distilled water. The bead contained thermo-stable polymerase (AmpliTaq™ DNA polymerase and Stoffel fragment), dNTPs (0.4 mM each),

BSA (2.5 μg) and buffer (3 mM MgCl$_2$, 30 mM KCl and 10 mM Tris, pH 8.3). Five primers (GE Healthcare, UK) were used in this study. Each primer is a 10-mer of arbitrary sequence: RAPD Analysis Primer 1 (P1) (5'-d[GGTGCGGGAA]-3'); RAPD Analysis Primer 2 (P2) (5'-d[GTTTCGCTCC]-3'); RAPD Analysis Primer 3 (P3) (5'-d[AAGAGCCCGT]-3'); RAPD Analysis Primer 4 (P4) (5'-d[AACGCGCAAC]-3') and RAPD Analysis Primer 5 (P5) (5'-d[CCCGTCAGCA]-3'). PCR was performed using a Veriti 96-well thermal cycler (Applied Biosystems, USA). PCR conditions included 1 cycle of 95°C for 5 min, followed by 45 cycles of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min. A long (20 × 14 cm) 1% agarose gel using 1X TAE buffer containing 0.5 μg/mL ethidium bromide was used for electrophoresis of the products. Gel images were obtained using Proxima C16 Phi+ (Isogen Life Science) UV transilluminator and Opticom (version 3.2.5; OptiGo) imaging system. Gel image analysis of the RAPD bands obtained for the plant species using different RAPD primers was performed using an Amersham 100-bp ladder (GE Healthcare) and the TotalLab TL100 1D software (version 2008.01).

## Conclusion

We primarily identified the endangered plant species on the basis of morphology. The combination of rbcL gene sequence and RAPD profile of *C. graveolens* subsp. *arabicum* may amend the identification process and help the conservation and tackling illegal trade of this regionally endangered species. As morphology based taxonomic methods require the whole plant, preferably in flowering stage for its authentic identification, DNA sequence may offer identification of the species even if a trace of tissue is available. Phylogenetic position or relationship of this species with other taxa must be drawn with caution as only few sequences of the related taxa are currently available in the databases. However, the presence of rbcL and other plant gene sequences in the databases has been increasingly fast. Therefore, future work on this species will provide reliable information regarding the phylogeny of this species.

## Acknowledgements

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