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Molecular Characterization of Regionally Endangered Tree Species *Mimusops laurifolia* (Forssk.) Friis (Sapotaceae)

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Abstract

Mimusops laurifolia (Forssk.) Friis is the largest tree species in the Arabian Peninsula and a characteristic species of the threatened 100 individuals that currently remained only at eleven localities. We determined the rbcL (ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit) gene sequence, current phylogenetic status and RAPD (Random Amplification of Polymorphic DNA) fingerprinting pattern of this regionally endangered plant species. Overall rbcL gene sequence similarity among the 18 plant species under the family Sapotaceae was very high (99.8%). The plastid rbcL gene sequence of *M. laurifolia* was identical to that of *M. zeyheri* indicating that *M. laurifolia* and *M. zeyheri* are very closely related plant species. *M. laurifolia* also showed close phylogenetic relation with *Argania spinosa* (99.6%). The RAPD profiles of *M. laurifolia* generated amplified products ranging from 517 to 1664 bp. A total of 11 bands were observed for *M. laurifolia* for 3 RAPD-primers. Morphological taxonomy of *M. laurifolia* and it's differential characters with similar plant species *M. zeyheri* are also briefly discussed in this study.

Keywords: *Mimusops laurifolia,* rbcL, RAPD

1. Introduction

Mimusops laurifolia (Forssk) Friis, (Synonym, *Binectaria laurifolia* Forssk., *M. schimperi* A. Rich.; Sapotaceae) (Friis, 1980) is the largest tree species in the Arabian Peninsula. It is native to Yemen, Saudi Arabia, Ethiopia and Somalia (Friis, 1980; Friis, 1992). Recently, this plant regained popularity since the extract was patented for skin-conditioning and moisturizing effects as part of preparations used in cosmetics, bath formulations and detergents (Ohara et al., 2001). The seeds are known to produce nine kinds of saponins (Eskander et al., 2006). *M. laurifolia* is a characteristic species of the threatened 100 individuals that are known to remain only at eleven localities. Many of these are extremely old trees with little or no regeneration. Attempts to multiply this slow growing tree have been so far failed (Kilan et al., 2004). Recently, these conditions are such a rarity in southwest Arabia that any future new records of *M. laurifolia* are likely to be of isolated adult tree which may not regenerate anymore. *M. laurifolia* was classified as endangered in the region on the IUCN red list (Hall et al., 2010). *M. laurifolia* is very less studied plant species. Plastid rbcL is the most commonly sequenced gene for phylogenetic studies of plants (Schuettpelz et al., 2006) and RAPD provides very quick and easy molecular characterization of taxa with some limitations (Penner et al., 1993). In this study, we determined the rbcLgene sequence based phylogenetic status and RAPD fingerprinting profile of this regionally endangered plant species. Descriptions of morphological characters of *M. laurifolia* are also briefly discussed in this study.

2. Materials and Methods

This study comprised of a specimen (specimen 4, voucher KSU 21544; Figure 3) taken from a tree in Al-Baha, Saudi Arabia (19.8653679, 41.3029594). The leaf samples were individually placed in plastic pouches and transported to the laboratory where the specimen was stored at -80^{o}C, until processed for DNA extraction. The plant was identified on the basis of morphological characters by Dr. Jacob Thomas, Curator, Herbarium, KSU. The

leaf specimen was immersed in liquid nitrogen and crushed using sterile mortar and pestle to get 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 -80^{o}C. Primer pair R1 (rbcLaF, 5′ATGTCACCACAAACAGAGACTAAAGC3′ (Levin, 2003); rbcLaR 5′GTAAAATCAAGTCCACCRCG3′ (Kress and Erickson, 2007) and R2 (rbcL1F, 5ATGTCACCACAAACAGAAAC3 (Fay et al., 1997); rbcL724R, 5′TCGCATGTACCTGCAGTAGC3′ (Fay et al., 1997) were used in this study for the amplification of rbcL gene of the chloroplast region. A total volume of 30 µL of PCR reaction mixture contained the following: 15 µL of FideliTaq PCR Master Mix {(USB Corporation, Cleveland, OH), giving a final concentration of 200 µM each deoxynucleotide and 1.5 mM $MgCl_2$}, 1 µM each primer (Eurofins MWG Operon, Germany), 2 µL of genomic DNA and the rest adjusted with sterile distilled water. PCR amplification was performed with a Veriti 96 well thermal cycler (Applied Biosystems) as follows: 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, T°C (51^{o}C for the primer pair R1 and 48^{o}C for the primer pair R2) for 30 s and 68°C for 1 min, followed by an elongation step at 68°C for 5 min. A long (20 x 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 pairs were determined by using an Amersham 100-bp ladder (GE Healthcare) and the TotalLab TL100 1D software (version 2008.01). Sequences were determined directly using the dideoxynucleotide chain-termination method with DNA-sequencer (ABI PRISM 3130xl; Applied Biosystems/Hitachi) and BigDye Terminator version 3.1 cycle sequencing RR-100 kit (Applied Biosystems), according to the manufacturer's instructions. Sequences were submitted to DDBJ/EMBL/GenBank database (Accession no. 4R1, JQ304270; 4R2, JQ304271). BLAST (Basic Local Alignment Search Tool) and BOLD (The Barcode of Life Data Systems) searches were applied to the produced sequences using the available online databases. Sequences of rbcL that were matched with the query sequences were retrieved from GenBank database. The sequences were aligned using CLUSTAL X (version 1.81) (Thompson et al., 1997). Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2007). Phylogenetic trees were constructed using Maximum Parsimony (MP, Close-Neighbor-Interchange algorithm; Nei and Kumar, 2000), Maximum Likelihood (ML; Tamura & Nei, 1993) and Neighbor-Joining (NJ; Saitou & Nei, 1987) methods. The topologies of the phylogenetic trees were evaluated by using the bootstrap resampling method (Felsenstein, 1985) with 1000 replicates.

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). Three primers (P1, P3 & P6; GE Healthcare, UK) were used in this study. Each primer is a 10-mer of arbitrary sequence: P1 (5′-GGTGCGGGAA-3′), P3 (5′-GTAGACCCGT-3′) and P6 (5′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 x 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).

3. Results

We obtained about 648 (4R1) and 814 bp (4R2) of PCR products of the rbcL gene of the plastid region of the species *M. laurifolia* using two sets of primer. Database search was conducted using the produced sequences. BLAST search with 4R1 showed the specimen (*M. laurifolia*) is identical (100% sequence similarity) to *M. zeyheri.* Search with 4R2 showed very close relationship (99% sequence similarities) with multiple plant species (*Argania spinosa*, EU980805; *Monotheca buxifolia*, AF421097; *Pouteria reticulata*, GQ428632). Recently developed BOLD identification system search of 4R1 showed 100% sequence similarities with multiple plant species under the genus *Mimusops* (*M. caffra, M. obovata, M. commersoni* and *M. zeyheri*). BOLD search with 4R2 showed 99.6% sequence similarities with *Argania spinosa* and *Monotheca buxifolia*. All the plant species that showed high level of sequence similarities (>99%) in the database search (genBank and BOLD) were included in the tree analyses. In general, all the inferred trees (MP, ML and NJ) showed similar topology.

According to all the tree analyses *M. laurifolia* was identical to that of *M. zeyheri* indicating that *M. laurifolia* and *M. zeyheri* are closely related plant species. *M. laurifolia* also showed close phylogenetic relation with *Argania spinosa* however, demonstrated a separate lineage from *A. spinosa* (supported by 80%, 65% and 67% of bootstrap values for MP, ML and NJ trees, respectively) (Figure 1; Supplementary Figures 1 & 2). Pair-wise sequence similarities of *M. laurifolia* were 100% with *M. zeyheri* and 99.6% with *A. spinosa* (Table 1). Overall average sequence similarity was observed very high (99.8%) among 18 plant species under the family Sapotaceae that were included in the tree analyses. All the rbcL gene sequence based phylogenetic trees demonstrated that different plant species under the family Sapotaceae are very closely related except *Diploon cuspidatum* and *Pradosia ptychandra* (Figure 1; Supplementary Figures 1 & 2).

Table 1. Estimates of pair-wise rbcL gene sequence similarities (%) among the studied taxa

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	99.6																	
3	**100**	99.6																
4	**100**	99.6	**100**															
5	99.6	**100**	99.6	99.6														
6	99.4	99.8	99.4	99.4	99.8													
7	99.6	**100**	99.6	99.6	**100**	99.8												
8	99.6	**100**	99.6	99.6	**100**	99.8	**100**											
9	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**										
10	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**									
11	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**								
12	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**	**100**							
13	99.4	99.8	99.4	99.4	99.8	99.6	99.8	99.8	99.8	99.8	99.8	99.8						
14	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**	**100**	**100**	99.8					
15	99.6	99.6	99.6	99.6	99.6	99.4	99.6	99.6	99.6	99.6	99.6	99.6	99.4	99.6				
16	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**	**100**	**100**	99.8	**100**	99.6			
17	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**	**100**	**100**	99.8	**100**	99.6	**100**		
18	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**	**100**	**100**	99.8	**100**	99.6	**100**	**100**	
19	99.6	**100**	99.6	99.6	**100**	99.8	**100**	**100**	**100**	**100**	**100**	**100**	99.8	**100**	99.6	**100**	**100**	**100**

1. *Mimusops laurifolia* specimen 4R1, 2. *Manilkara zapota*, 3. *Mimusops laurifolia* specimen 4R2, 4. *Mimusops zeyheri*, 5. *Manilkara huberi*, 6. *Diploon cuspidatum*, 7. *Micropholis cayennensis*, 8. *Pouteria singularis*, 9. *Pouteria gongrijpii*, 10. *Chrysophyllum sanguinolentum*, 11. *Pouteria reticulate*, 12. *Manilkara bidentata*, 13. *Pradosia ptychandra*, 14. *Pouteria filipes*, 15. *Argania spinosa*, 16. *Monotheca buxifolia*, 17. *Sideroxylon inerme*, 18. *Madhuca microphylla*, 19. *Palaquium formosanum*. Identical sequence similarities (100%) were typed in bold.

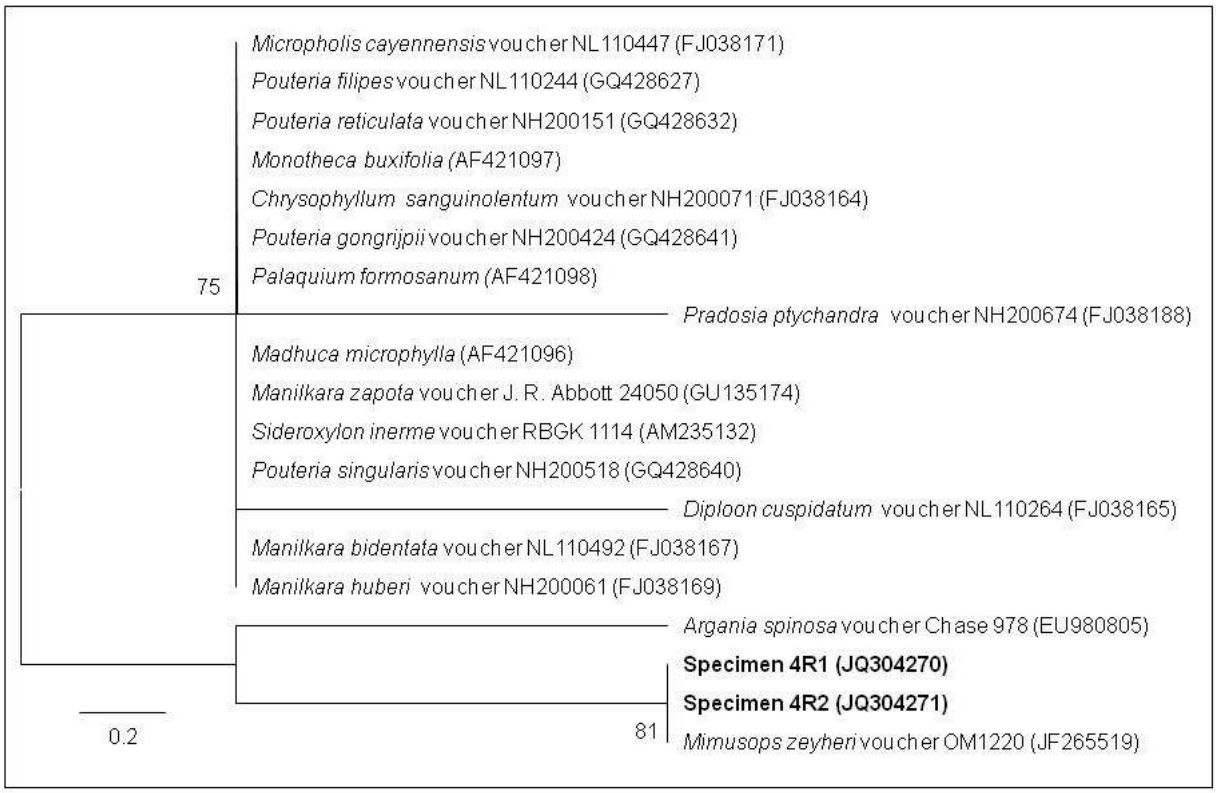


Figure 1. Maximum Parsimony tree inferred from the rbcL gene sequences of the studied specimens, *Mimusops laurifolia* and related taxa

The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The scale represents the units of the number of changes over the whole sequence. GenBank accession numbers are written in parentheses.

The RAPD banding pattern of the endangered plant species *M. laurifolia* is illustrated in Figure 2. The RAPD profiles using the sample generated amplified products ranging from 517 to 1664 bp. A total of 11 bands were observed for the plant species using 3 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 P6 (5 bands) and the equal number of bands for P1 and P3 (3 bands, Figure 2).

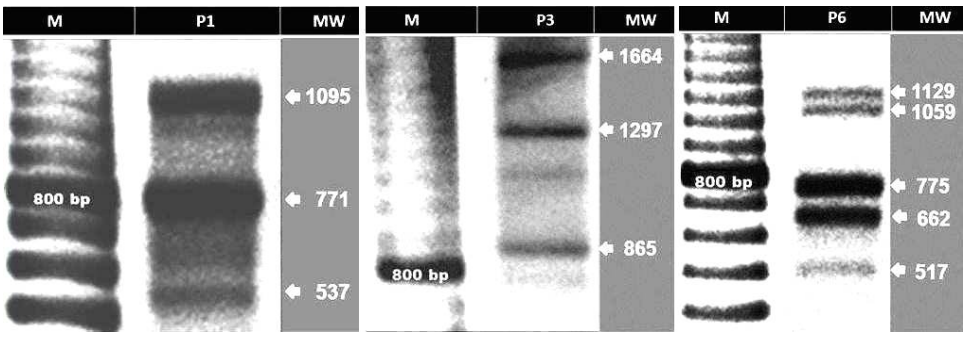


Figure 2. RAPD-PCR product profile of the regionally endangered plant *M. laurifolia*. M, 100-bp molecular weight marker; P, Ready-To-Go RAPD analysis primers (P1, P3 & P6; GE Healthcare, Buckinghamshire, UK); MW, Molecular weight

4. Discussion

M. laurifolia showed very close relationship with *M. zeyheri* based on the rbcL gene sequence phylogeny. Morphologically *M. laurifolia* is closely related with *M. zeyheri* and previously this species was classified as a variety of *M. zeyheri* (*M. zeyheri* var. *laurifolia*; Engler, 1904). Literature on the description of taxonomy of *M. laurifolia* is very scarce and typified with a single specimen found in the Forsskäl herbarium (Friis, 1980). *Mimusops laurifolia* (Forssk.) Friis and *M. zeyheri* (Sond.) are extremely similar morphologically (Figures 3 & 4) with notable few differences that are illustrated in Table 2.

Table 2. Morphological comparison of *M. lurifolia* and *M. zeyheri*

Characters	*M. laurifolia*	*M. zeyheri*
Color of tender branches	Brown or green	Pink or crimson
Tree trunk	Buttresses present	Buttresses absent
Pubescence	Young braches glabrous or thinly pubescent	Young branches densely pubescent, covered with light reddish colored hairs
Petiole	Petiole of mature leaves 4-5 cm long and young leaves 3-4 cm long	petiole 0·6–3·5 cm long
Pedicel (in flower)	1-2.5 cm long	1-1.7 cm long
Style	1.5-2.5 mm long	5-9 mm long
Distribution	Southwestern Arabian Peninsula and north eastern Africa	Angola-South Africa and Tanzania

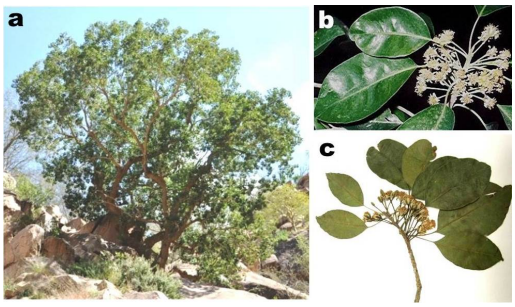
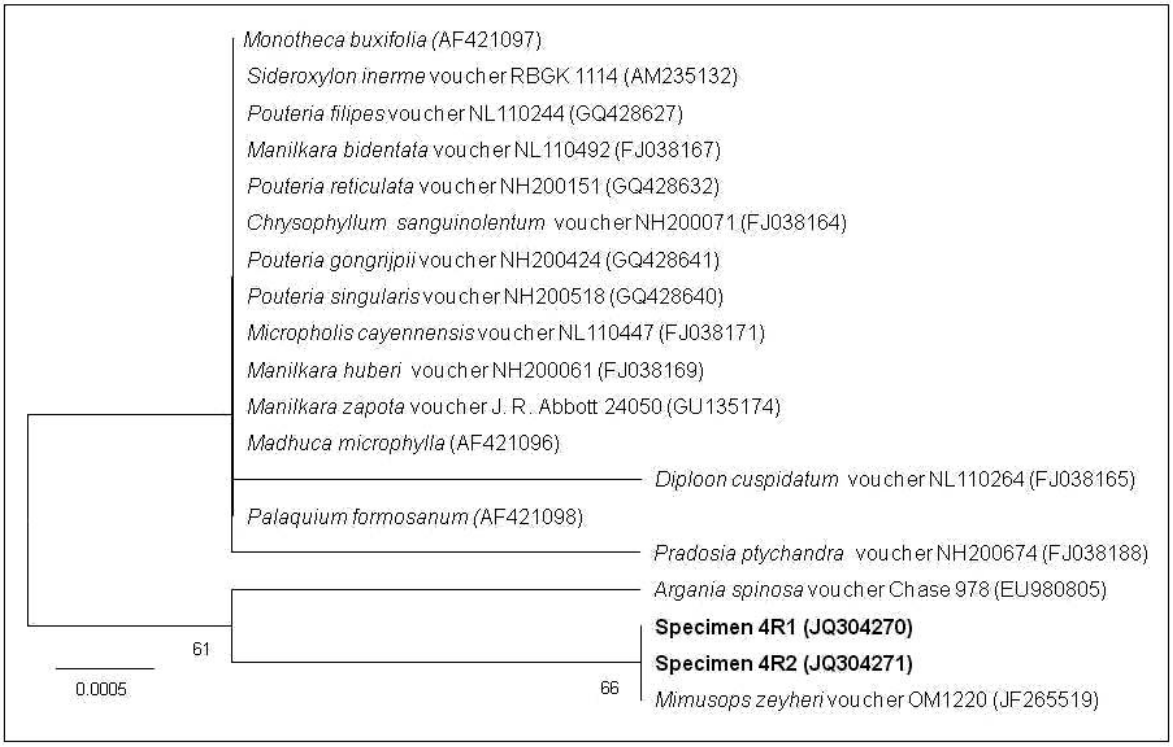


Figure 3. a. *M. laurifolia* tree; b. Leaf and flower; c. Herbarium specimen of *Mimusops laurifolia* (KSU 21544)

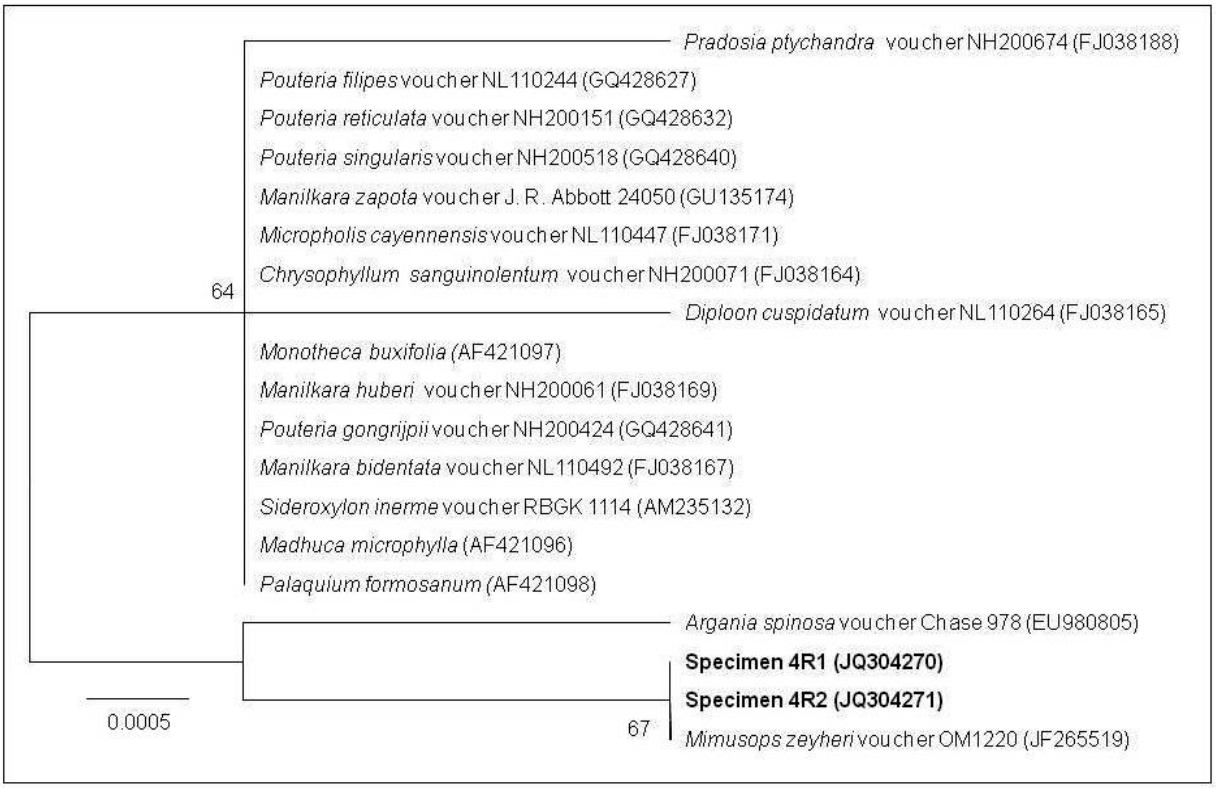
Figure 4. a. *Mimusops zeyheri* tree; b. Leaf; c. Flower; d. Fruit. Adapted from Lemmens (2005); Land plants (2007); The African Expedition Magazine (2012)

M. zeyheri is distributed in the area of Tanzania to South Africa (Bisby et al., 2011). *M. laurifolia* is distributed in south western wadis and lower heights of Saudi Arabia, tropical East Africa and Yemen (Chaudhary, 1999). Species-descriptions of *M. laurifolia* include it as a high, large-trunked tree and grow up to 30 m tall. Leaves are usually crowded towards the ends of branches; petiole can be observed up to 4 cm long; lamina broadly elliptical (± 9 x 4.5 cm), entire obtuse. Flowers are solitary axillary, long pedicelled; pedical can be up to 20 mm. Sepals densely tomentose; outer sepal usually 7 mm long, inner sepal a little smaller. Petals are ± 9 mm long. Fruits are oval yellowish green in color, berry (4 x 2.5 cm), edible and sweetish (Chaudhary, 1999). Plastid rbcL is the most commonly sequenced gene for phylogenetic studies of plants (Schuettpelz et al., 2006) because of the other plant marker such as matK is difficult to amplify and sequence (Bafeel et al., 2011; Hollingsworth, 2011). Previous study based on rbcL gene sequence showed that *Glycyrrhiza uralensis* was identical to that of *G. inflata*, indicating that *G. uralensis* and *G. inflata* are closely related species (Hayashi et al., 2000). RAPD primers are able to characterize taxa (Choo et al., 2009) because RAPD analysis includes both of the coding and non-coding regions of the genome (Vanijajiva et al., 2005). However, 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). It is suggested that 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 & Nienhuis, 1995).



Supplementary Figure 1. Maximum Likelihood tree inferred from the rbcL gene sequences of the studied specimens and the related taxa

The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The scale represents the units of the number of changes over the whole sequence. GenBank accession numbers are written in parentheses.



Supplementary Figure 2. Neighbor-Joining tree inferred from the rbcL gene sequences of the related taxa with the studied specimen

The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The scale represents the units of the number of changes over the whole sequence. GenBank accession numbers are written in parentheses

5. Conclusions

Plastid rbcL gene based phylogeny showed *M. laurifolia* is very closely related with *M. zeyheri* and very close relationship among the other morphologically defined taxa under the family Sapotaceae. Development of markers with high discriminatory power at species level is necessary to clarify the phylogenetic status of the different plant species under the family Sapotaceae.

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