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ORIGINAL ARTICLE

Identification and Molecular Phylogeny of Morphologically of the Medicinal Plant *Calligonum L'Herit*

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ABSTRACT

Aim: The rare gem species of *Calligonum comosum* contains highly therapeutic compounds collected from Al-Qassim region, Saudi Arabia. It has various biological properties that used for antimicrobial, antioxidant, anti-inflammatory, anti-gastric ulcer, anti-hyperglycemic, abdominal ailments as well as against toothache.

Methods: *Calligonum* L. (*Polygonaceae*) genus consists of 158 species dispersed in Asia, Africa and Middle East. Remarkably a very few researches found molecular phylogeny of *Calligonum comosum*, it is one of the significant medicinal plants used in various herbal preparations and most commonly adulterated by the morphologically similar species, *viz C. ebinuricum.* The species *C. junceum, C. Colubrinum* and *C. arborescens* is often misidentified as *Calligonum comosum* hence, thereby accurate identification of this medicinal herb are very crucial which ensures the drug effectively as well as biosafety. Thus, in this study, DNA barcoding tool was used for precise identification, its conservation development and differentiating from analogous species of *C. comosum*.

Results: The barcode genes like nuclear internal transcribed spacer region ITS2, the chloroplast plastid gene *matK* and *rbcL* were used for phylogenetic analysis of *Calligonum* species. From the sequence alignment the nuclear internal transcribed spacer ITS2 regions shows 9.5% of polymorphic sites, indicates higher range of transition/transversion (ts/tv) ratio and percentage of variation which aids to discriminate *Calligonum comosum* from its closely related species.

Conclusion: The present phylogeny proves that the nuclear internal transcribed spacer ITS2 region distinguishes the closely related species of *Calligonum comosum* from its morphologically similar species.

Keywords: *Calligonum*, Phylogeny, ITS2, matK, rbcL.

INTRODUCTION

Calligonum L. Her (*Polygonaceae*) genus consists of 158 species distributed in the regions of India, China, North Africa, Pakistan, Afghanistan, Saudi Arabia and South Europe[1]. *Calligonum comosum* L'Hert (*Erta* plant in Arabic) also known as *Escanbil* species which, are dominant shrubby bare psammophytes broadly distributed in mobile and semi-mobile sand dunes of Indian desserts, Middle Asia, the Middle east, Iran and Saudi Arabia[2,3]. The *Erta* plant *Calligonum comosum* L'Hert is a huge perennial, enduring fuzzy shrub has glaucous woody stem with greyish-white color, firm branches. It was characterized by bushy shrub that grows up to 3 m tall and the stems were lignified woody with white branches. These branches are woody, furry with swollen nodes and long internodes. The leaves are 1.5 mm in size, long clustral inflorescence, and silvery-white flowers with sweet smelling. It's fruit size is about 12-13 mm across. *Calligonum comosum* lacks a main trunk but has rigid, lignified basal white branches, but upper young branches are green and thin with very small Caducous leaves[4]. Slivery white colored flowers which spring during the month of March and April. It has perianth[4], the fruits covered by long hairs which arising from four vertical wing-like narrow ridges. The plant has a long tap root which enables the plant to pull together the sand and is used as sand dune stabilizer[5]. Researchers report that the species *Calligonum comosum* has thicker vessel walls with long and narrow vessel elements and fibers that help in adapting it in hot deserts[6].

Calligonum comosum L' Herit, subspecies of *C. polygonoides L*. that can quickly have distinguished by small leaves, white flowers, larger nuts, and pedicels are shorter with equal distribution of the perianth lobe. Researcher[4] studied about the eco-physiological characteristics of *Calligonum comosum* under different levels of drought stress. It cures scabies and stomach ailments. The stems and leaves are chewed for curing toothache[7]. Species of *Calligonum comosum* from Abu Dhabi Emirates found to be rich in anti-oxidant, anti-inflammatory, antiulcer, antidiabetic activity and cytotoxic properties[8,9]. It had proven to have various biological properties that in turn had antimicrobial, antioxidant, anti-inflammatory, anti-gastric ulcer, anti-hyperglycemic activities with abdominal ailments as well as against toothache Researchers[8,9,10] studied estrogenic and antimicrobial activities and treatment towards gynecological disorder found promising[11]. Reported about *C. comosum* had antimicrobial property against food-contaminating bacteria belonging to *Listeria* genus. The volatile constituents were extracted by hydro distillation from the aerial parts of *C. comosum, C. azel* and *C. arich* were observed at the flowering stage[12]. The extract of *C. comosum* extract inhibits carcinogenesis when tested in diethylnitrosamine-induced hepatocarcinogenesis in rats[13,14].

In the present study, the chloroplast genes such as *rbcL, matK*, and nuclear internal transcribed spacer ITS2 region are used as barcode candidates. The nuclear ITS2 regions accurately identify and distinguish the *Calligonum comosum* from the other morphologically alike species of *Calligonum*. The entire DNA barcode candidate differentiates the six species of *Calligonum*.

METHODS

Plant sample collection: The plant specimen collected from Al-Qassim region, Saudi Arabia had its GPS Latitude and Longitude coordinates as 26.251914^0 , 43.660726^0 respectively as shown in Figure1.a. The specimen was identified by Taxonomist, at Department of Biology, College of Arts and Sciences, Al - Baha University.

DNA extraction, PCR amplification and DNA sequencing: Total genomic DNA was extracted from fresh leaves using modified CTAB method (Doyle and Doyle, 1987). The chloroplast genes like *matK, rbcL* and nuclear internal transcribed spacer ITS2 region were amplified using previously reported primers. The PCR products were resolved in 1 % agarose gel using 0.59 TBE buffer.

Sequence alignment and phylogenetic analysis: The sequence of similar species was obtained from GenBank, NCBI and constructing sequence alignments with phylogenetic antiquities along with molecular evolutionary analysis was done by MEGA 6.0[14]. CLUSTAL W program was used for multiple sequence alignment (Thompson et al., 1994). Phylogenetic analysis based on maximum likelihood method (ML) and Kimura 2-Parameter (K2P) model were used to find genetic distance and variation. GTR model were used to weigh the informative characters and Markov Chain Monte Carlo (MCMC) method for tree sampling using MEGA[14].

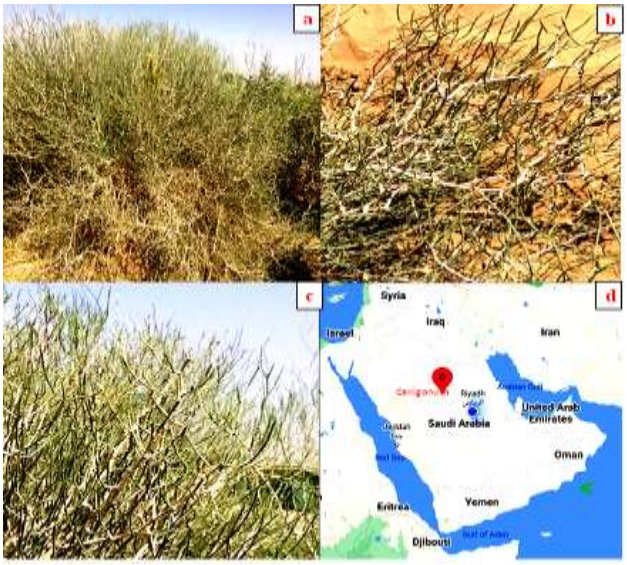

Figure 1. a (a, b, c) The rare gem plant specimens were collected from Al-Qassim region, Saudi Arabia (GPS coordinate: 26.251914^0 , 43.660726^0 , Adopted from https://maps.google.com). d. The map of collected site.

RESULTS

Inter versus intra-specific genetic divergence: The aligned length of *matK, rbcL* and nuclear internal transcribed spacer ITS2 sequences was 590, 507 and 463 respectively. Three metrics like interspecific, intraspecific and mean distance, were employed to characterize specific variations in the genus of *Calligonum* (Table-I). The chloroplast *matK* shows 0.95 % of interspecific distance, 0.28% of intraspecific distance and 0.37% of mean distance respectively. The chloroplast gene *rbcL* represents 1.36% interspecific distance, 0.08% of intraspecific distance, 0.63% of mean distance and the nuclear spacer region shows 4.43% of interspecific distance, 0.43% of intraspecific distance and 1.91% of mean distance.

Table-I: Summary of DNA sequences

Parameters	*matK*	*rbcL*	ITS2
Number of sequences (retrieved from GenBank)	31	22	15
Length (bp)	590	507	463
Interspecific distance (%)	0.95	1.36	4.43
Intraspecific distance (%)	0.28	0.08	0.43
Mean distance (%)	0.37	0.63	1.91

Identification based on DNA sequence similarity: BLAST program was used for species identification through markers the *matK, rbcL* and nuclear transcribed region ITS2.The gene *matK*, *rbcL* and *ITS2* showed 98.92%, 99.07% and 99.84 % of similarities respectively (in Table-II), that represents the order of identification of the species at Genus level.

Table-II: Precise Identification of species using nuclear and chloroplast loci.

Marker	Methods	Similarity (%)	% of variation
matK	BLAST	98.92	0.113
rbcL		99.07	0.031
ITS2		99.84	0.580

Tree based classification: In the previous literature study, *Fallopia multiflora* were selected from Polygonaceae family proposed as the sister to *Calligonum*[15] similarly *Pteropyrum* were selected which have been proposed as the clade sister to *Calligonum*. Other than similarity-sequence based identification, phylogenetic tree was drawn using *matK, rbcL* and ITS2 region. Phylogenetic analysis was carried out for 11 genera of *Calligonum,* 2 species of *Pteropyrum* and *Atraphaxis suaedifoli* as an out group. The Akaike Information Criterion (AIC) scores about 21,029.691 using MEGA. The Kimura 2-Parameter and gamma distribution methods used for boot strapping and Maximum Likelihood (ML) method used for inferring the phylogenetic tree.

Phylogeny using *MatK* gene: Nearly 34 sequences were collected for the matK gene among those 26 species are of *Calligonum* and 8 different isolates of *Fallopia.* The outgroup species of *Fallopia* were distributed in the separate clade. The *Calligonum* consist of 6 different species. The matK gene of *C.comosum* is closely related with the species *C. ebinuricum.* The species *C. junceum, C. Colubrinum* and *C. arborescens* are closely related to each other. The species *C. korlaense, C. ebinuricum, C. junceum* and *C. aphyllum* are related to each other in separate clade. Thus, each taxon of *Calligonum* for matK was clearly distinguished as in the Figure1.

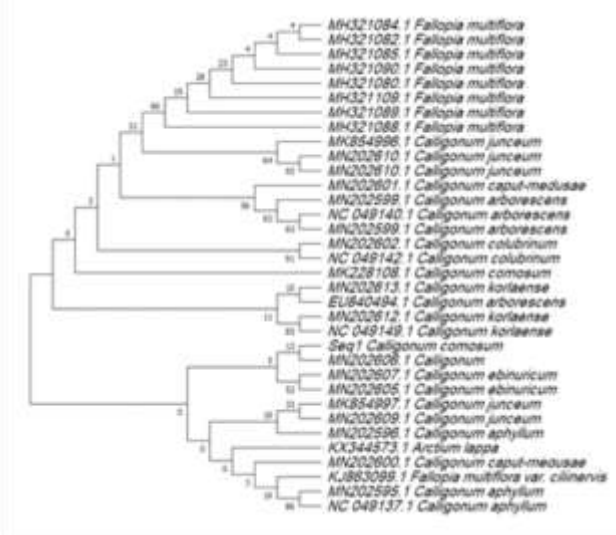

Figure 1. Maximum Likelihood (ML) tree using *matK* gene sequences for the species of *Calligonum*.

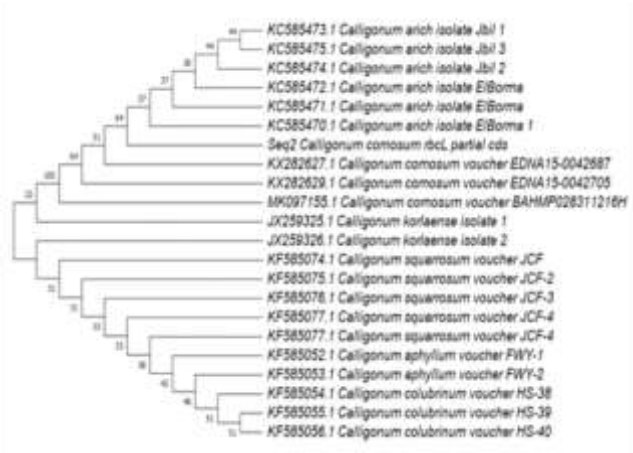

Figure 2. Maximum Likelihood (ML) tree using *rbcL gene* sequences for the species of *Calligonum*.

Phylogeny using *rbcL* gene: The species *Calligonum arich isolates Jbil1, isolates Jbil3* are likely as sister taxa similarly to *C. colubrinum voucher HS-39, HS-40.* The species suchas *Calligonum arich isolate Jbil2, Calligonum arich isolate ElBorma, Calligonum arich isolate ElBorma 1, C. korlaense isolate 1, 2, C. squarrosum, C. aphyllum* are more closely related sharing common ancestor as shown in the Figure 2.

Phylogeny using ITS2: Thus, all the above results represent that, nuclear ITS2 region distinguishes the 11 plant species of the genus

Calligonum. The five species *C. junceum*, *C. arborescens, C. crinitum, C. comosum* and *C. polygonoide*s are distributed in the same clade. The species *C. persicum* and *C. bungei* are closely related and as separate clade. The out-group species *P. olivierii* and *P. naufelum* as separate clade. The species *C. squarrosum* and *A. suaedifolia* as monoclade, respectively as in Figure 3.

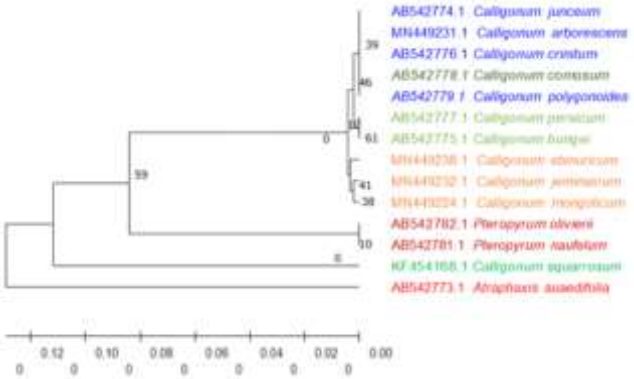

Figure 3. Maximum Likelihood (ML) tree using ITS2 sequences for the species of *Calligonum.*

DISCUSSION

By means of, as DNA was extracted, PCR amplification with the universal primers of chloroplast genes like *matK*, *rbcL* and nuclear ITS2 spacer region and the amplicons were sequenced and submitted to GenBank. Multiple sequence alignment and pairwise sequence alignment analysis were performed using chloroplast gene *matK*, *rbcL* and nuclear internal transcribed *ITS2,* respectively. From the sequence analysis, the gene *rbcL* shows 0.08% of intraspecific distance and the size was also found to be slightly longer, which made this gene unsuitable for barcoding purpose. The other gene *matK* and *ITS2,* showed 0.28 and 0.43 % of intraspecific distance, respectively. Based on considerable variation from the conserved gene sequence used to distinguish and differentiate the species. The result strongly reports that the chloroplast gene *matK and rbcL* distinguishes at genus level but the nuclear spacer region ITS2 distinguishes at species level of *comosum.* Although *matK, rbcL,* and ITS2 regions have good amplification efficiency and mentioned by[16,17] that using BLAST method, three barcode candidate *matK*, *rbcL,* and *ITS2* clearly differentiate the species. From the phylogenetic analysis, the genes *matK*, and *rbcL* distinguishes the species at genus level and mostly find to be monophyletic. But by using *ITS2* region it clearly distinguishes the species and reported that it successfully discriminates the Asteraceae species reported by[18], Apocynaceae described by[19,20,21] and in the universal barcodes in plants and animals identification proved by[22] and ITS2 in tribulus species mentioned by[23].

CONCLUSION

Thus, the study determines and accurately identifies the species *Calligonum comosum* from its morphologically similar species. Thereby, the study results underwent the phylogenetic analysis and genetic variations that existed in the genera *Calligonum* as it has rich medicinal properties. The accuracy, time and cost-effectiveness can be achieved by DNA-based identification techniques overcome the shortcoming seen in morphological and chemical analysis. The present investigation proves that the nuclear internal transcribed spacer ITS2 region distinguishes the closely related species of *Calligonum comosum*.

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