DNA Barcoding of two endangered medicinal Plants from Abou Galoom protectorate

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Abstract: DNA barcoding is a recent and widely used molecular-based identification system that aims to identify biological specimens, and to assign them to a given species. However, DNA barcoding is even more than this, and besides many practical uses, it can be considered the core of an integrated taxonomic system, where bioinformatics plays a key role. DNA barcoding data could be interpreted in different ways depending on the examined taxa but the technique relies on standardized approaches, methods and analyses. We tested two medicinal endangered plants (*Cleome droserifolia* and *Iphiona scabra*) using two DNA barcoding regions (ITS *and rbcL*). The ITS and *rbcL* regions showed good universality, and therefore the efficiency of these loci as DNA barcodes. The two loci were easy to amplify and sequence and showed significant inter-specific genetic variability, making them potentially useful DNA barcodes for higher plants. The standard chloroplast DNA barcode for land plants recommended by the Consortium for the Barcode of Life (CBOL) plant working group needs to be evaluated for a wide range of plant species. We therefore tested the potentiality of the ITS and *rbcl* markers for the identification of two medicinal endangered species, which were collected from Abou Galoom protectrate, South Sinai, Egypt. Wild plants belonging to diverse families of arid regions. Maximum likelihood tree analysis was performed to evaluate the discriminatory power of the ITS and *rbcL* genes. In this work ITS and *rbcl* markers were used to discriminate and confirm the identification of two medicinal endangered plants, it was found that, the viability and potentiality of ITS region in identification process for the two plants used is more efficiency than *rbcl*, where *rbcl* confirm the identification of two plants at generic level, while ITS at the species level. There is also four new sequences were obtained from using each previous marker, two new sequences for *C. droserfolia* and another two for *I. scabra*.
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Key Words: DNA barcoding; ITS; *rbcl;* medicinal endangered plants; Identification.

1. Introduction:

DNA barcoding is a molecular tool that uses a short locus from a standardized genome position to provide fast and accurate species identification (http://www.barcoding.si.edu). This technique is helpful in taxonomic, ecological, and evolutionary studies. In addition, it can be used in more applied fields (e.g. conservation, forensic science, and the food industry) and can enable the accurate identification of crypticspecies (**e.g. Lahaye *et al*., 2008; Ragupathy *et al*., 2009).**

The term ‘‘DNA barcode’’ is used here to refer to a DNA sequence-based identification system that may be constructed of one locus or several loci used together as a complementary unit **(Kress and Erickson, 2007).**

The most important characteristic features of a DNA barcode are its universality, specificity on variation and easiness on employment. This means that the gene segment used as a barcode should be suitable for a wide range of taxa, should have high variation between species but should be conserved within the species, so that the intra-specific variation will be insignificant **(Kress *et al*., 2005, Pennisi, 2007, CBoL, 2009 and Viayan and Tsou, 2010).**

DNA barcoding in plants seems to be inherently more difficult than in animals **(Chase *et al*., 2005; Pennisi, 2007; Fazekas *et al*., 2009).** Several different loci, and combinations of there, have been suggested as suitable barcodes for land plants **(e.g. Pennisi, 2007; Ford *et al*., 2009).** For instance, the nuclear internal transcribed spacer (ITS) region and the plastid intergenic spacer *trnH-psbA* have been proposed for flowering plants **(Kress *et al*., 2005),** whereas the latter has been suggested for land plants in general **(Chase *et al*., 2005).** Other suggested loci include *rbcL* **(Newmaster *et al*., 2006),** the chloroplast *trnL* intron **(Taberlet *et al*., 2007),** and three regions proposed by Ki- Joong Kim, *atpF-atpH*, *matK*, and *psbK-psbI* **(Pennisi, 2007).** Recently, the two-locus combination of *rbcL+matK* has been recommended as the core barcode for land plants **(CBOL plant Working Group, 2009).**

Many DNA markers have been tested to elucidate the Phylogenetic relationships among bryophytes especially mosses as cleared by **Quandt**

and Stech (2003). Many of the phylogenetic markers proposed were used recently as DNA Barcoding markers to help in identification of difficult taxa (**El-Sakaty *et al.*, 2014)**.

Molecular characters are primarily obtained from three different sources: (i) DNA sequences of specific coding or non-coding regions from one of the three plant genomes (plastid, mitochondrial, or nuclear markers), (ii) structural genomic characteristics (e.g. gene order, gain or loss of genes, or non-coding regions), and (iii) genetic fingerprints (**Stech and Quandt, 2010).**

DNA sequences from organelle genomes (e.g. mitochondria, chloroplasts) have been widely used for reconstructing phylogenetic relationships (**Lin *et al.*, 2002**). They are widely considered to be uniparentally-inherited and non-recombining with a single shared evolutionary history for the entire organelle genome (**Wolfe and Randle, 2004**).

One of the most widely used regions in the phylogeny of plants is *rbcL* (Ribulose -1,5 – bisphosphate carboxylase/ oxygenase large subunit gene) which responsible for the production of the large subunit of the enzyme RuBisCo (involved in the first major step of carbon fixation).

The plastid *rbcL* gene is certainly the most sequenced locus among land plants and has also been extensively sequenced for bryophytes. However, in bryophyte molecular systematics *rbcL* seems to be less popular than in ferns or angiosperms. One reason is the rather low sequence variation at family level and below that soon became evident in early studies and indicated that multigene analyses are required to corroborate the findings (**Goffinet *et al.*, 1998; De Luna *et al.*, 2000; Maeda *et al.*, 2000; Tsubota *et al.*, 2001; Forrest *et al.*, 2006 and Bell *et al.*,). 2007; Heinrichs *et al.*, 2005, 2007 and Wahrmund *et al.*, 2010**) and is not suitable for species and population level analysis or barcoding approaches in bryophytes. This contrasts with views of **Newmaster *et al.* (2006)** and **Liu *et al.* (2010)**, who considered *rbcL* as the marker with the best performance as DNA barcode in bryophytes.

Internal Transcribed Spacer (ITS) is one of the most used polymorphic regions is, a space of non-coding RNA situated between structural ribosomal RNAs on a common precursor transcript. ITS spacer is known to be partitioned into ITS1 and ITS2 separated by 5.8S ribosomal cistron (fig. 2), in which the RNA poly-cistronic precursor transcript will be in this order 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S (**Wheeler and Honeycutt, 1988**). As a part of the transcriptional unit of rDNA, the ITS spacers 1 and 2 are therefore present in all organisms (**Calonje *et al.*, 2009**).

Since their first application by **Porter and Collins (1991)**.ITS1 and ITS2 are widely used for phylogeny reconstruction, due to the following reasons stated by many early studies (e.g. **Baldwin *et al.*, 1995; Liston *et al.*, 1996 and Maggini *et al.*, 1998):** 1- Biparental inheritance: in comparison to the maternally inherited chloroplast and mitochondrial markers. 2- Easy PCR amplification with several universal primers available for various kinds of organisms. 3- Multi-copy structure, which can be found in up to a few thousand copies *per* cell. 4- Moderate size, which allows reasonable sequencing. 5- Based on published studies the variation at the level that makes it suitable for evolutionary studies at the species or generic level **(Poczai and Hyvonen, 2010)**.

Although ITS region proved to be useful marker, several problems such as flaws in the concerted evolution mechanism, the existence of paralogs and orthologs and the presence of pseudogenes were reported (**Mayol and Rosselló, 2001; Bailey *et al.*, 2003; Feliner and Roselló, 2007 and Soltis *et al.* 2008).**

Cleome droserifolia, family Cleomaceae, grows in South Sinai, Egypt **(Boulos 2000)**.It is endangered wild plant **(Abd El- Wahab *et al.*, 2004).** It also has a long history of medicinal use, especially in Sinai for the treatment of DM in individuals with non-insulin dependent diabetes **(Ismael, 1992)**. It has hypoglycemic properties as it significantly suppressed the rise in peripheral blood glucose concentrations in albino rats **(Ismael *et al*., 1996).** The methanol extract of *C. droserifolia* has two flavonoids as active components **(Fushiya 1999).**

. The role of *C. droserifolia* in increasing insulin levels could be secondary to its property as an antioxidant (**Ismael *et al.*, 1996**). Therefore, *C. droserifolia* could have a protective effect on pancreatic cells against oxidative stress-induced cellular damage, which certainly affects the synthetic capacity of these cells. However,it can be suggested that *C. droserifolia* extract may exert antioxidant activities that protect the tissues from destructive damage of lipid peroxidation (**El-Shenawy and Abdel-Nabi 2004**) and is unlikely to be due to the stimulation of pancreatic β-cells and subsequent secretion of insulin. Finally, *C. droserifolia* extract not only exhibits hypoglycemic properties but also reduces oxidative stress in alloxan-induced diabetic mice and increases insulin release (**El-Shenawy and Abdel-Nabi 2006**).

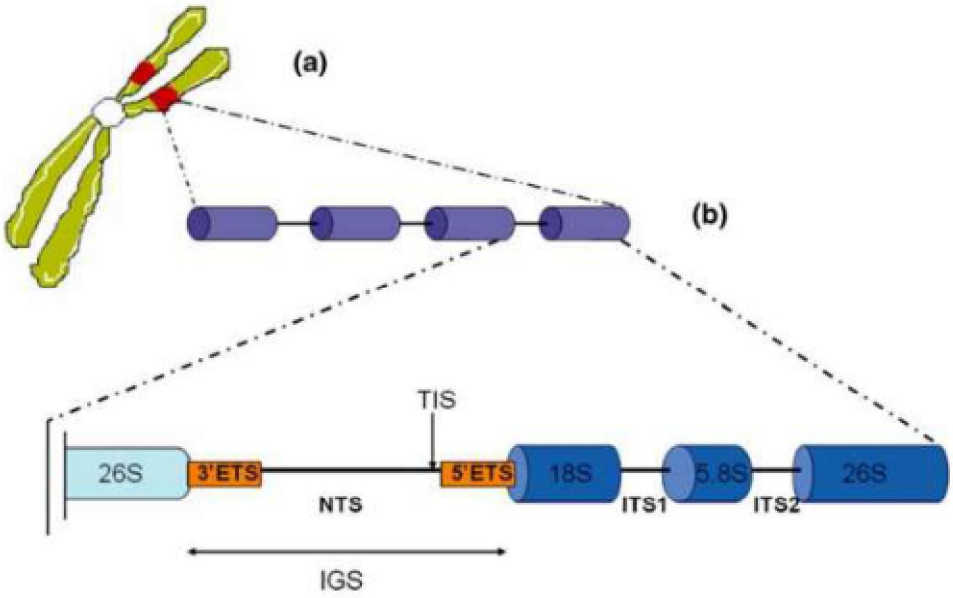

Figure 2. Schematic representation of the universal structure of the rDNA region inplants. (a) The chromosomal location of the rDNA regions. (b) Tandem arrays of the consecutive gene blocks (18S-5.8S-26S). In the tandem arrays each gene block is separated by an intergenic spacer (IGS) consisting of a 5′ end and 3′ end external transcribed spacer (ETS). The two regions are separated by a non-transcribed region (NTS). The transcription start site (TIS) labels the start position of the 5′ETS. The small subunit (18S) and large subunit genes (5.8S and 26S) are separated by the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2(ITS2).

I. scabra DC (Asteraceae: subfam. Inulae) **(Boulos 2000)**. It is medicinal endangered wild plant growing in the Sinai Peninsula **(Abd El-Wahab *et al*., 2004).** It is rich in coumarin and pyrrolizidine alkaloid and flavonoides.Thirteen flavonoides, quercetin, and pyrrolizidine alkaloid were isolated **(Ahmed and Tom** 1987**)**. *I. scabra* is used in traditional medicine as an antispasmodic drug **(Font-Quer, 1990).**

I. scabra extract has anticoagulant, anti-platelet aggregation and anti-inflammatory effects in carrageenan-induced rat paw oedema. Moreover, the mean blood pressure significantly lowered by administration of the aqueous extracts of *I scabra* when compared with nefidepine treatment (hypotensive standard drug) in a dose dependant manner (**Nada *et al*., 2006)**

Sharaby *et al*., 2014 found that ethanolic extracts of *Iphiona scabra* caused the maximum inhibition of egg hatchability and caused the highest depression in the deposited eggs, as they played a remarkable role as ovipositor deterrents.

It was found that, the universality of barcode markers is hampered due to morphological/geographical variation and reticulate evolution in plant species (**Roy *et al*., 2010**). The ongoing research on plant barcoding suggests that the development of universal DNA barcoding markers for land plants is challenging; even the choice of the correct loci has been debated (**Chase *et al*., 2005; Kress *et al*., 2005; Fazekas *et al*., 2008; de Groot *et al*., 2011**). Arguments about the selected core loci for plant barcoding are related to the lack of discriminatory power and/or primer universality (**Roy *et al*., 2010**). Plant species of the desert are adapted to tolerate multiple stresses, including high extremes of drought, temperature, solar radiation, wind, and salinity (**Batanouny, 2001).**

The phylogenetic studies were firstly based on the sequence of one DNA locus. The advanced tools in the molecular studies and confused results obtained from only one locus encourage many researchers for using multiple loci based analysis. Each locus differs in its rate of evolution according to many factors as place and coding or non-coding. It was clearly found that the nuclear DNA has more variation and higher rate of evolutionary steps than chloroplast DNA and the latter is more evoluted in plants than the mitochondrial DNA.

The aim of this work is to discriminate and confirm the identification of two endangered medicinal plants at the molecular level, using ITS and *rbcl* regions. Then confirm their morphological identification through reference sequences in database and GenBank. Also determine viability and

potentiality, of the two regions used in the identification process of the two samples, finally record any new gene(s).

2. Material and Methods

Samples collection

Green leaves of two endangered medicinal plants, were collected from from Abou Galoom protectorate, south Sinai, Egypt and were identified by Dr: El-Sayeda Gamal El-Deen prof of taxonomy, Botany Dept. Suiz Canal Univ.

DNA extraction

Dried plant leaf samples from two endangered species were used. DNA extraction was carried out using SIGMA® Plant High Molecular DNA extraction KIT®, Plant tissue was disrupted by grinding in liquid nitrogen and DNA was released with detergent and chaotropic agents. Proteins, polysaccharides, and cell debris were eliminated with a 10 minute precipitation procedure followed by centrifugation through a filtration column, included in the kit. The genomic DNA was purified further by a silica bind-wash-elute procedure in micro-centrifuge spin columns. DNA quality was tested using agarose gel electrophoresis, visualized by pre-added RedSafe® (5ul/100ml) under UV light and quantified using Eppendorf® Spectrophotometer X100 device, about 50µg of DNA were obtained from 2g ground powder of dry plant material.

PCR and sequencing

Two primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the internal transcribed spacer (ITS) according to **White *et al.* (1990).** While the primer pairs rbcLaF (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3') and rbcLaR (5'-GTA AAA TCA AGT CCA CCR CG-3') were used to amplify *rbcL* region. PCRs of 50 ul reaction mixture (1X Flexi buffer, 50ng DNA template, 2.5mM MgCl2, 10uM dNTPs, 0.4uM of each primer, and 1U Promega© Green Go Taq™ enzyme) were performed, standard PCR profile with 55ºC annealing temperature was used to amplify ITS and 50ºC to amplify *rbcL*. Results were tested on 1.5% agarose gel electrophoresis and visualized by pre-added 1x RedSafe® using a UV light. When successful, amplified fragments were cleaned and concentrated using Thermo GeneJET PCR Purification Kit #K0702. Cleaned fragments were sequenced by private service (Macrogen, Netherlands). Sequence chromatograms were compiled using Bioedit V3 to assemble the sequences. All sequences were manually aligned, while gaps inserted to preserve nucleotide homology. Ambiguous regions were deleted from the analyses. All Haplotype sequences were submitted into the GenBank database (http://www.ncbi.nlm.nih.gov; accessions KR**998497** – KP**998498** for **ITS** for **C**. ***droserifolia*** and ***I***. ***scabra*** respectively and KR9984919 & KR998500 for ***rbcL***).

Molecular identification, assignment of taxa and phylogenetic analysis

Wide range of studies targeted BLASTn tool to identify samples based on nucleotide sequence, nevertheless such identification does not consider the evolution model nor supported with a significance test. For such, the BLASTn tool were used to determine the candidates for a supported phylogenetic analysis using several methods. To identify the evolutive position and study phylogenetic relationships of the two endangered species, the aligned sequences were analyzed by maximum likelihood (ML) analysis implemented in MEGA6 **(Tamura *et al*., 2013)**. Tree inference options were set to Nearest Neighbor Interchange. Gaps/missing data were treated as partial deletions with site coverage cut off = 95%. A bootstrap analysis with 1000 replicates was carried out in order to study the clade support values. In all methods, trees were generated in the presence of the available ITS and *rbcL* related sequences found by BLASTn tool (NCBI). Analyses were conducted using the Maximum Composite Likelihood model. The rate variation among sites was modeled with a gamma distribution (Shape parameter = 0.48). The consensus tree was obtained after bootstrap analysis, with 1,000 replications, with values above 50% was reported. The analysis involved 22 and 26 nucleotide sequences for ITS and *rbcL*, respectively.

3. Results and Discussion

Morphological identification

Based on the morphological aspect, the two endemic samples that were collected from St. Katherine (Abou Galoom) protectorate have been identified as follows:

Sample no. (1) is *Cleom droserifolia,*while sample no.(2) is *Iphiona scabra.*

Molecular Identification and DNA Barcoding

Using both regions (ITS and *rbcL*), the phylogenetic analysis was performed using both samples together with GenBank accessions. Both trees were rooted between both samples that revealed two main clads, each clade possessed one sample per se and belong to a certain family.

ITS phylogenetic analysis, showed that, sample 1 does belong to the family Cleomeace, and specifically to genus Cleome. Sample 1 was highly supported to *Cleome droserifolia* (bootstrap support of 87%). Sample 2 does belong to the family Asteraceae, and specifically to genus *Iphiona.* Sample 2 was fully supported to *Iphiona scabra* (bootstrap support of 100%) (Fig. 2).

rbcL phylogenetic analysis, showed that, sample 1 does belong to the order Brassicales, and specifically to genus Cleome. Sample 1 was poorly supported to *Cleome spp.* (bootstrap support of 37%). Sample 2 does belong to the family Asteraceae, and specifically to tribe Inulinea. Sample 2 was fairly supported to *Inulia spp.* (bootstrap support of 50%) (Fig. 3).

By comparing between both sequenced regions, the nuclear ITS region found to have a better resolution toward species identification than the *rbcL* sequences and this result agree with **Kress *et al*., 2005 and Chase *et al*.,2005.** As Cleomeace family belong to order Brassicales, while *Iphonia* spp. belongs to tribe Inulinea of the Astercea family. That might be due to the insufficient *rbcL* similar sequences of this two species in the GenBank database, or the *rbcL* marker is not the most suitable to be applied for the DNA barcoding for such families. Even though the ITS was more efficient, it cannot be relied on as a single DNA barcoding region due to its variation within a single species (e.g. *Funaria hygrometrica*, **Magdy, 2013**) or due to the presence of paralogs, orthologs and pseudogenes of ITS sequence in a single genome (**Nieto Feliner & Rosello, 2007and Soltis *et al.*, 2008**). However, by combining both markers, the two collected endangered medicinal plant samples from St. Katherine (Abou Galoom) area were identified with high support as *Cleome droserifolia* (sample 1) and *Iphonia scabra* (sample 2).

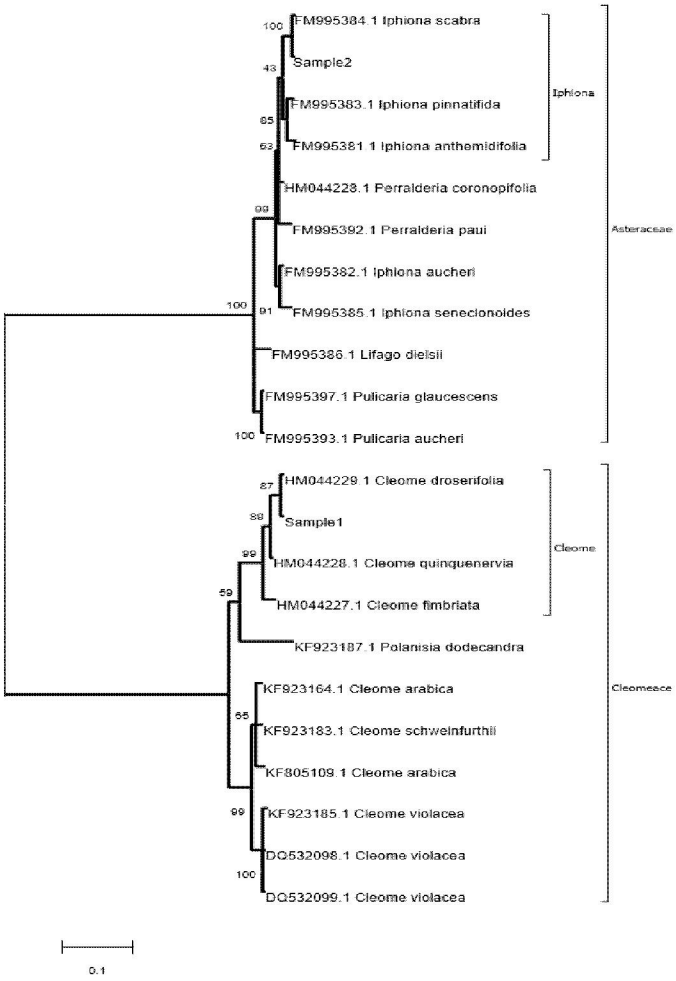

Figure 2: ITS based maximum likelihood tree for both collected samples. Two main clades each belong to a different families were distinguished. Sample 1 was highly supported with *Cleome droserifolia*, while sample 2 was fully supported with *Iphonia scabra*.

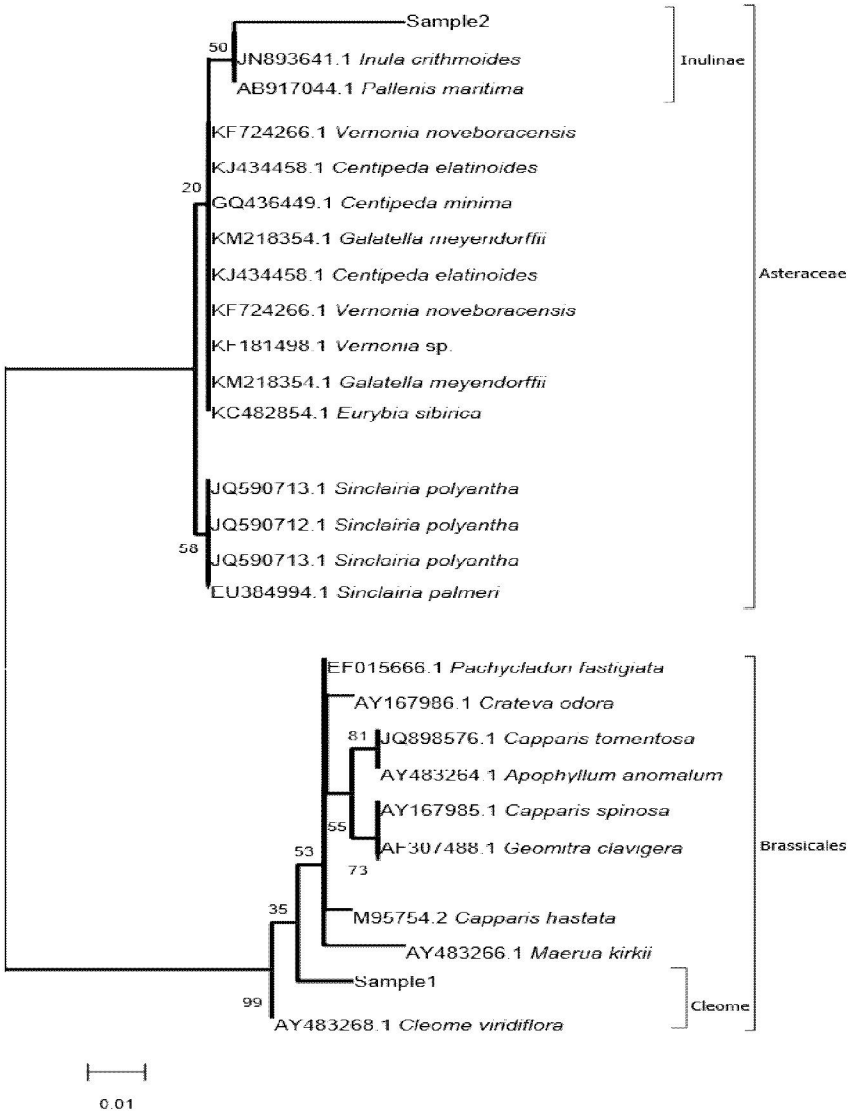

Figure 3: *rbcL* based maximum likelihood tree for both collected samples. Two main clades each belong to a different families were distinguished. Sample 1 was supported with *Cleome spp.*, while sample 2 was supported with tribe Inulinae.

In conclusion, this study provides preliminary assessment data that will be useful for wider application of DNA barcoding in medicinal endangered wild plants. With the current development of primers, we found that ITS will be very useful for the barcoding of some medicinal endangered plant species, where it has a better resolution toward species identification than the *rbcL* sequences. However, further protocol development to enhance clean DNA extraction, PCR amplification strategies, including the development of new primers, and local authenticated databases could play important roles in efficient utilization of plant barcoding.

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