

DNA Barcoding of two endangered medicinal Plants from Abou Galoom protectorateH. El-Atroush¹, M. Magdy² and O. Werner³¹ Botany Department, Faculty of Science, Ain Shams University, Abbasya, Cairo, Egypt.² Genetics Department, Faculty of Agriculture, Ain Shams University, 68 Hadayek Shubra, 12411, Cairo, Egypt.³ Departamento de Biología Vegetal, Facultad de Biología, Universidad de Murcia, Campus de Espinardo, 30100, Murcia, Spain.Hala_elatroush@hotmail.com

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. droserifolia* and another two for *I. scabra*.

[H. El-Atroush, M. Magdy and O. Werner. **DNA Barcoding of two endangered medicinal Plants from Abou Galoom protectorate**. *Life Sci J* 2015;12(9):101-109]. (ISSN:1097-8135). <http://www.lifesciencesite.com>. 14

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 Wahrmond 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**).

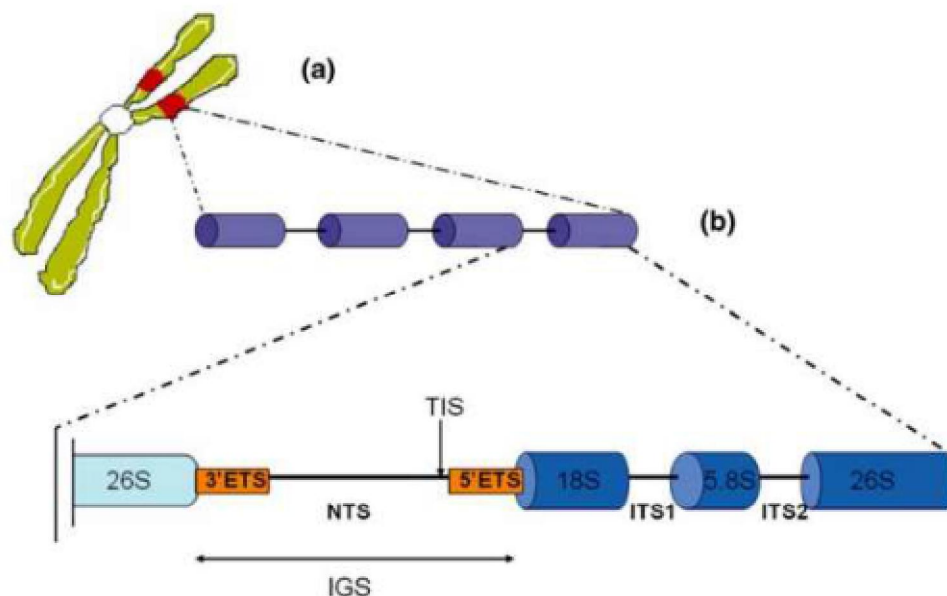


Figure 2. Schematic representation of the universal structure of the rDNA region in plants. (a) The chromosomal location of the rDNA regions. (b) Tandem arrays of the consecutive gene blocks (18S-5.8S-26S). In the tandem arrays each gene block is separated by an intergenic spacer (IGS) consisting of 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 nefedipine 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 evolved 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 55°C annealing temperature was used to amplify ITS and 50°C 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 KR998497 – KP998498 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 *Cleome 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, 2007** and **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).

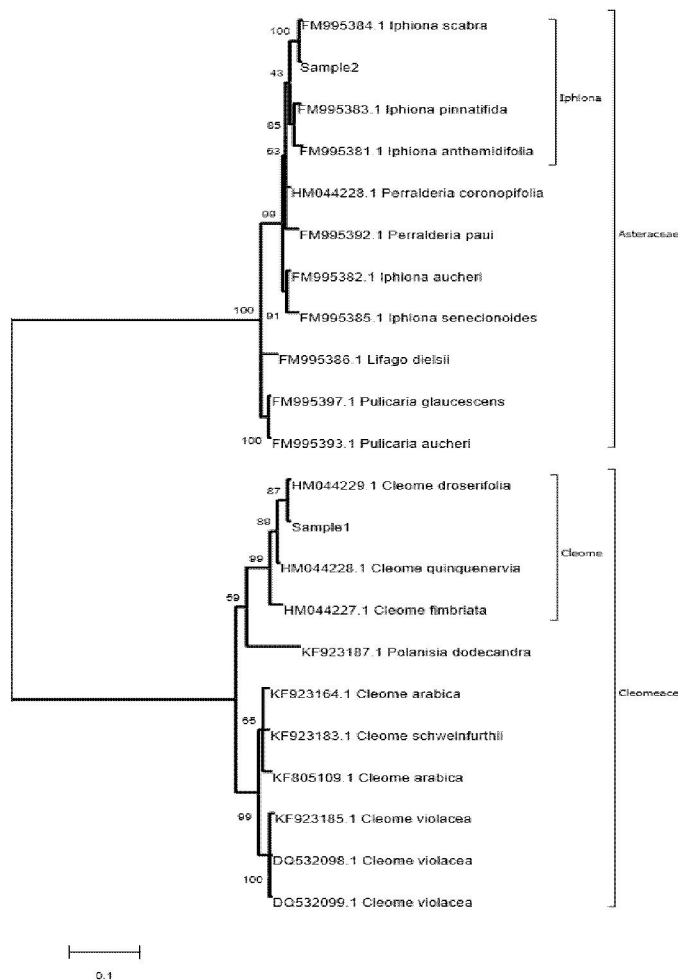


Figure 2: ITS based maximum likelihood tree for both collected samples. Two main clades each belong to a different families were distinguished. Sample 1 was highly supported with *Cleome droserifolia*, while sample 2 was fully supported with *Iphonia scabra*.

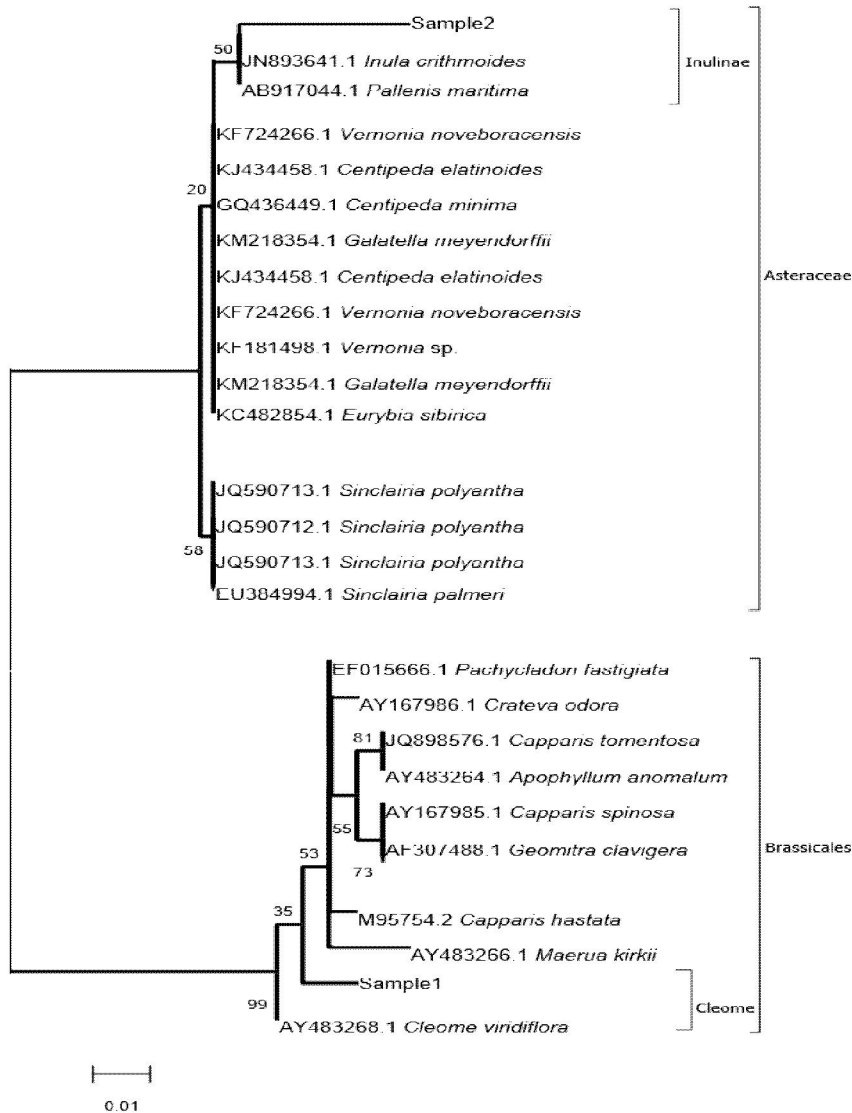


Figure 3: *rbcL* based maximum likelihood tree for both collected samples. Two main clades each belong to a different families were distinguished. Sample 1 was supported with *Cleome spp.*, while sample 2 was supported with tribe Inulinae.

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.

References

1. Abd El-Wahab, R.H.; Zaghloul, M.S. and Mostafa, A.A. 2004. Conservation of medicinal plants in St Katerine protectorate, south Sinai, Egypt. Proceeding first international conference on strategy of Egyptian Herbaria. March 9-11; Giza, Egypt.
2. Ahmed, A.A. and Tom, J. Mabry Flavonoids of *Iphiaona scabra*. *Phytochemistry* 1987;26:1517-8.
3. Bafeel, S.O.; Arif I.A.; Bakir M.A.; Al Homaidan, A.A.; Al Farhan, A.H. and Khan H 2012: DNA barcoding of arid wild plants using *rbcL* gene sequences, *Genet. and molecular Research* 11(3):1934-1941.

4. Bailey, C.D.; Carr, T.G.; Harris, S.A. and Hughes, C.E. 2003. Characterization of Angiosperm nrDNA polymorphism, paralogy, and pseudogenes. *Mol. Phyl. Evol.* 29, 435–455.
5. Batanouny, K.H.2001. Plants in the Deserts of the Middle East. Springer, New York.
6. Baldwin, B.G.; Sanderson, M.J.; Porter, J.M.; Wojciechowski M.F., Campbell, C.S. and Donoghue, M.J. 1995. The ITS region of nuclear ribosomal DNA. a valuable source of evidence on angiosperm phylogeny. *Ann. Mis. Bot. G.* 82, 247–277.
7. Bell, N.E; Quandt, D.; Ó'Brien, T.J. and Newton, A.E. 2007. Taxonomy and phylogeny in the earliest diverging pleurocarps. square holes and bifurcating pegs. *Bry.* 110, 533–560.
8. Boulos, L.2000. (Flora of Egypt), volume two, pp.177-179, Al-Hadara publishing, Cairo, Egypt.
9. Borsch, T. and Quandt, D. 2009. Mutational dynamics and phylogenetic utility of noncoding chloroplast DNA. *Pl. Sys. Evol.* 282, 169–199.
10. CBOL Plant Working Group 2009. A DNA barcode for land plants. *Proc. Natl. Acad. Sci. U. S. A.* 106: 12794-12797.
11. Calonje, M.; Martín-Bravo, S.; Dobes, C.; Gong, W., Jordon– Thaden, I.; Kiefer, C.; Kiefer, M., Paule, J.; Schmickl, R. and Koch, M.A. 2009. Non-coding nuclear DNA markers in phylogenetic reconstruction. *Pl. Sys. Evol.* 282, 257–280.
12. Chase, M.W.; Salamin, N.; Wilkinson, M.; Dunwell JM, *et al.* 2005. Land plants and DNA barcodes: short-term and long-term goals. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360: 1889-1895.
13. de Groot, G.A.; During, H.J.; Maas, J.W.; Schneider, H, *et al.* 2011. Use of *rbcL* and *trnL-F* as a two-locus DN, A barcode for identification of NW-European ferns: an ecological perspective. *PLoS One* 6: e16371.
14. De Luna, E.; Buck, W.R.; Akiyama, H.; Arikawa, T., Tsubota, H.; Gonzalez, D.; Newton, A.E. and Shaw, A.J. 2000. Ordinal phylogeny within the Hypnobryalean pleurocarpous mosses inferred from cladistic analyses of three chloroplast.
15. DNA sequence data sets. *trnL-F*, *rps4*, and *rbcL*. *Bry.* 103, 242–256.
16. Duffy, A.M.; Kelchner, S.A. and Wolf, P.G. 2009. Conservation of selection on *matK* following an ancient loss of its flanking intron. *Gene* 438: 17-25.
17. El-Sakaty, S. I. A.; Magdi, M.; El-Atroush, H., Mohamed, M. M.; Abou-Salama, U. 2014. DNA Barcoding of three Bryaceae –Musci. *Egypt. J. Bot.*, special issue for the fourth international conference of Botany and Microbiology Sciences. 53–63.
18. El-Shenawy, N.S. and Abdel-Nabi, I.M.2006 : Hypoglycemic Effect of *Cleome Droserifolia* Ethanolic Leaf Extract in Experimental Diabetes, and on Nonenzymatic Antioxidant, Glycogen, Thyroid Hormone And Insulin Level Diabetologia Croatica 35-1, 2006 15.
19. El-Shenawy, N.S. and Abdel Nabi I.M.2004. Comparative analysis of the protective effect of melatonin and *Cleome droserifolia* extract on antioxidant status of diabetic rats. *Egypt J Hosp Med* 2004;14:11-25.
20. Fazekas AJ; Burgess KS; Kesanakurti PR; Graham SW, *et al.* 2008. Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. *PLoS One* 3: e2802.
21. Fazekas, A.J.; Kesanakurti, P.R.; Burgess, K.S., Percy, D.M.; Graham, S.W.; Barrett, S.C.H; Newmaster, S.G.; Hajibabaei, M.; Husband, B.C. 2009. Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? *Molecular Ecology Resources* 9(s1): 130–139.
22. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.
23. Feliner, G.N. and Rosselló, J.A.2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS inspecies-level evolutionary studies in plants. *Mol. Phyl. Evol.* 44, 911–91.
24. Font-Quer, P. 1990. Plantas medicinales, el Dioscorides Renovado, 12th ed., Spain Editorial Labor, SA, Barcelona.
25. Ford, C.S.; Ayres, K.L.; Toomey, N.; Haider, N.; Stahl, J.V.; Kelly, L.J.; Wikstrom, N.; Hollingsworth, P.M.; Duff,R.J.; Hoot, S.B.; Cowan, R.S.; Chase, M.W.; Wilkinson, M.J. 2009. Selection of candidate coding DNA barcoding regions for use on land plants. *Botanical Journal of the Linnean Society* 159: 1–11.
26. Fushiya, S.; Kishi, Y.; Hattori, K., *et al.* Flavonoids from *Cleome droserifolia* suppress NO production in activated macrophages *in vitro*. *Planta Med* 1999;65:404-407.
27. Goffinet, B.; Bayer, R.J. and Vitt, D.H. 1998. Circumscription and phylogeny of the Orthotrichales Bryopsida. Inferred from *rbcL* sequence analysis. *Am. J. Bot.* 85, 1324–1337.
28. Hebert, P.D. and Gregory, T.R. 2005. The promise of DNA barcoding for taxonomy. *Syst. Biol.* 54: 852-859.
29. Heinrichs, J.; Gradstein, S.R.; Wilson, R. and Schneider, H. 2005. Towards a natural classification of liverworts Marchantiophyta. based on the chloroplast gene *rbcL*. *Cryp. Bry.* 26, 131–150.
30. Heinrichs, J.; Hentschel, J.; Wilson, R.; Feldberg, K. and Gradstein, S.R. 2007. Evolution of leafy liverworts Jungermanniidae, Marchantiophyta. Estimating divergence times from chloroplast DNA sequences using penalized likelihood with integrated fossil evidence. *Taxon* 56, 31–44.
31. Hollingsworth, M.L.; Andra C.A.; Forrest, L.L.; Richardson J, *et al.* 2009. Selecting barcoding loci for plants: evaluation of seven candidate loci with

- species-level sampling in three divergent groups of land plants. *Mol. Ecol. Resour.* 9: 439-457.
32. Hershkovitz, M.A.; Zimmer, E.A. and Hahn, W. 1999. Ribosomal DNA sequences and Angiosperms. In: Hollingsworth, P.M., Bateman, R.M. and Gornall, R.J. Eds.
 33. *Molecular Systematics and Plant Evolution*. Taylor and Francis, London, pp. 268–326 <http://www.barcoding.se.gdu> <http://www.ncbi.nlm.nih.gov>.
 34. Ismael, L.D. 1992. Pharmacognostical study of certain Cleome species growing in Egypt. Master of Science thesis. Faculty of Pharmacology, Al-Azhar University.
 35. Ismael, W.G.; Ibrahim, K.M.; Mikhail, T.H. *et al.* Role of the hypoglycemic plant extract *Cleome droserifolia* in improving glucose and lipid metabolism and its relation to insulin resistance in fatty liver 1996. *Boll. Chim. Farm.* 135:507-551.
 36. Kew, British Royal Botanical Garden website, accessed 20/5/2014.
 37. Kress, W.J. and Erickson, D.L. 2007. A two-locus global DNA for land plants. the coding *rbcL* gene complements the non-coding *trnh-psbA* spacer region. *PLoS ONE* 2, e508.
 38. Kress, W.J.; Wurdack, K.J.; Zimmer, E.A.; Weigt, L.A. and Janzen, D.H. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. USA* 102, 8369–8374.
 39. Lahaye, R.; Van Der Bank, M.; Bogarin, D.; Warner, J., Pupulin, F., Gigot, G., Maurin, O.; Duthoit, S.; Barraclough, T.G. and Savolainen, V. 2008. DNA barcoding the floras of biodiversity hotspots. *Proceedings of the National Academy of Sciences, USA* 105: 2923–2928.
 40. Lin, Y.; Mclenachan, P.; Gore, A.; Philips, M.; Ota, R. and Hendy, M. 2002. Four new mitochondrial genomes and the increased stability of evolutionary trees of mammals from improved taxon sampling. *Mol. Biol. Evol.* 19, 2060–2070.
 41. Liston, A.; Robinson, W.A.; Oliphant, J.M.; Alvarez-Buylla, E.R. 1996. Length variation in the nuclear ribosomal DNA internal transcribed region of non-flowering seed plants. *Syst. Bot.* 21 2, 109–120.
 42. Liu, Y.; Yan, H.F.; Cao, T. and Ge, X.J. 2010. Evaluation of 10 plant barcodes in Bryophyta Mosses. *J. Syst. Evol.* 48, 36–46.
 43. Maeda, S.; Kosuge, K.; Gonzalez, D.; DeLuna, E. and Akiyama, H. 2000. Molecular phylogeny of the suborder Leucodontineae Musci, Leucodontales. Inferred from *rbcL* sequence data. *J. Pl. Res.* 113, 29–38.
 44. Magdy, M. 2013. *Genetic variability in mosses and its relation to climate change adaptation processes in Mediterranean environments*. Ph.D. thesis, Dep. Of plant biology, Murcia University.
 45. Maggini, F.; Marrocco, R.; Gelati, T.M.; De Dominicis, R.I. 1998. Length and nucleotide sequences of the internal spacers of nuclear ribosomal DNA in Gymnosperms and Pteridophytes. *Pl. Syst. Evol.* 213, 199–205.
 46. Mayol, M. and Rosello, J.A. 2001. Why nuclear ribosomal DNA spacers ITS. tell different stories in Quercus? *Mol. Biol. Evol.* 19, 167–176.
 47. Nada, A.; Mohey, S.A.; Ellithy, M.E and Khattab, A. 2006. Novel pharmacological properties of *Iphiona scabra* and *Jasonia montana* (Asteraceae) aqueous extracts: screening for anti-platelet aggregation, anti-inflammatory and hypotensive effects in albino rats. *The Egyptian Medical Journal of the National Research Centre*, December 2006, 5(2): 28-32.
 48. Nei, M. and Kumar, S. 2000. *Molecular Evolution and Phylogenetics*. Oxford university Press, New York.
 49. Newmaster, S.G.; Fazekas, A.J.; Ragupathy, S. 2006. DNA barcoding in the land plants: Evaluation of *rbcL* in a multigene tiered approach. *Can. J. Bot* 84: 335–341.
 50. Pennisi, E. 2007. Taxonomy Wanted, A barcode for plants. *Sci.* 318, 190–191.
 51. Plamer, D. and Delwiche, F. 1998. The origin and evolution of plastids and their genomes. In D. Soltis, J. Doyle and M. Norwell Eds. *Molecular Systematic of Plants*, II, DNA sequencing pp.375–409. Kluwer Academic.
 52. Poczai, P. and Hynven, J. 2010. Nuclear ribosomal spacer regions in plant phylogenetics. problems and prospects. *Mol. Biol. Rep.* 37, 1897–1912.
 53. Porter, C.H. and Collins, F.H. 1991. Species-diagnostic differences in the ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* Diptera. Culicidae. *Am. J. Trop. Med. Hyg.* 45, 271–279.
 54. Quandt, D. and Stech, M. 2003. Molecular systematics of bryophytes in context of land plant phylogeny. In: Sharma, A.K. and Sharma, A. Eds. *Plant Genome. Biodiversity and Evolution*, Vol. 1, Part A. Science Publishers, Enfield, New Hampshire, pp. 267–295.
 55. Ragupathy, S.; Newmaster, S.G.; Balasubramaniam, V. and Murugesan, M. 2009. DNA barcoding discriminates a new cryptic grass species revealed in an Ethnobotany study by the hill tribes of the Western Ghats in southern India. *Mol. Eco. Res.* 9, 164–171.
 56. Rogers, S.O. and Bendich, A.J. 1987. Ribosomal RNA genes in plants. Variability in copy number and in intergenic spacer. *Pl. Mol. Biol.* 9, 509–520.
 57. Roy, S.; Tyagi, A.; Shukla, V., Kumar, A., *et al.* 2010. Universal plant DNA barcode loci may not work in complex groups: a case study with Indian berberis species. *PLoS One* 5: e13674.
 58. Sharaby, A.; Abdel-Rahman, H. and Moawad, S. 2014 (A novel 6-hydroxy-8,11,11-trimethyl-bicyclo[7.2.0]undec-4-ene-4-carboxylic acid: a potent antioxidant agent from *Iphiona scabra*).

59. Soltis, D.E.; Mavrodiev, E.V.; Doyle, J.J., Rauscher, J. and Soltis, P.S. 2008. ITS and ETS sequence data and phylogeny reconstruction in allopolyploids and hybrids. *Sys. Bot.* 33, 7–20.
60. Stech, M. and Quandt, D. 2010. 20,000 species and five key markers: the status of molecular bryophyte phylogenetics. *Phytotaxa* 9, 196–228
61. Sugiura, C.; Kobayashi, Y.; Aoki, S.; Sugita, C. and Sugita, M. 2003. Complete chloroplast DNA sequence of the moss *Physcomitrella patens*: evidence for the loss and relocation of *rpoA* from the chloroplast to the nucleus. *Nuc. Acids Res* 31, 5324–5331.
62. Sugiura, M. 1992. The chloroplast genome. *Plant Mol. Biol.* 19, 149–168.
63. Spooner, D.M. 2009. DNA barcoding will frequently fail in complicated groups: An example in wild potatoes. *Am. J. Bot.* 96: 1177–1189.
64. Taberlet, P.; Coissac, E.; Pompanon, F.; Gielly, L., Miquel, C.; Valentini, A.; Vermet, T.; Corthier, G.; Brochmann, C. and Willerslev, E. 2007. Power and limitations of the chloroplast *trnL* UAA intron for plant DNA barcoding. *Nuc. Acids Res.* 35, e14.
65. Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* 10: 512–526.
66. Tamura, K.; Stecher, G.; Peterson, D.; Filipiński, A. and Kumar, S. 2013. MEGA6. Molecular Evolutionary Genetics Analysis V6.0. *Mol. Biol. Evol.* 30, 2725–2729.
67. Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F., *et al.* 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25: 4876–4882.
68. Tsubota, H.; Akiyama, H.; Yamaguchi, T. and Deguchi, H. 2001. Molecular phylogeny of the Sematophyllaceae Hypnales, Musci. based on chloroplast *rbcL* sequences. *J. Hattori Bot. Lab.* 90, 221–240.
69. Vanderpoorten, A.; Goffinet, B. and Quandt, D. 2006. Utility of the internal transcribed spacers of the 18S–5.8S–26S nuclear ribosomal DNA in land plant systematics, with special emphasis on bryophytes. In: Sharma, A.K. and Sharma, A. Eds., *Plant Genome. Biodiversity and Evolution*, Vol. 2, Part B. Science Publishers, Enfield, New Hampshire, pp. 385–407.
70. Viayan, K. and Tsou, C.H. 2010. DNA Barcoding in Plants: Taxonomy in a new perspective. *Current Sci* 99(11), 1530–1541.
71. White, T.J.; Bruns, T.; Lee, S. and Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White.
72. TJ eds. PCR protocols: a guide to methods and application. San Diego: Academic Press. 315–322.
73. Wheeler, W.C. and Honeycutt, R.L. 1988. Paired sequence difference in ribosomal RNAs. Evolutionary and phylogenetic implications. *Mol. Biol. Evol.* 5, 90–96.
74. Wolfe, A.D. and Randle, C.P. 2004. Recombination, heteroplasmy, haplotype polymorphism, and paralogy in plastid genes. Implications for plant molecular systematics. *Syst. Bot.* 24, 1011–1020.
75. Wahrmond, U.; Quandt, D. and Knoop, V. 2010. The phylogeny of mosses – Addressing open issues with a new mitochondrial locus. Group I intron *cobI420*. *Mol. Phyl. Evol.* 54, 417–426.
76. Yao, H.; Song, J.Y. and Liu, C. 2010. Use of ITS2 region as the universal DNA barcode for plants and animals. *PLoS ONE* 5, e13102.
77. Yan, LIU; Hai-Fei YAN; Tong CAO and Xue-Ju G, Evaluation of 10 plant barcodes in Bryophyta (Mosses) 2010: journal of Systematics and Evolution 48 (1): 36–46. doi: 10.1111/j.1759-6831.2009.00063.x.

9/22/2015