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New molecular and biochemical records for *Mindium laevigata* at its various developmental stages

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Abstract:

It is essential to identify and determine the properties of native plants as natural genetic resources. The present study was performed to identify the *Mindium* (*Michauxi) laevigata* species using molecular and biochemical procedures such as genomic DNA extraction, sequencing, and antioxidant capacity and protein content determination at both vegetative and generative phases in various parts of the plant. For this purpose, *Mindium laevigata* plants were collected from natural habitats and their genomic DNAs were extracted and purified. This was followed by the extraction of 18S ribosomal DNA sequence from the genomic DNA by PCR and its analysis to determine the antioxidant enzymes (peroxidase, ascorbate peroxidase, and catalase). Accordingly, the proteins were quantitatively and qualitatively assayed at both vegetative and reproductive stages in the different plant organs of roots, stems, and leaves. Ascorbate peroxidase and catalase activities were detected in the stem samples at the vegetative and generative phases, respectively. Gel electrophoresis bands of the total protein were found to be different in various parts and at different developmental stages of the plant. Another aspect of the study involved the use of the phylogenetic tree for the biosystematic investigations of *Mindium laevigata*. Molecular analyses resulted in the inscription of a new gene in GenBank under the accession number KC294445.1. *Mindium laevigata* seems to be a rich source of antioxidant enzymes and proteins and as such it is recommended for further research.

Keywords: Antioxidant enzymes; Biosystematics*; Mindium laevigata*; 18S Ribosomal DNA.

Introduction:

The Campanulaceae family consists of about 90 genera and 2500 species (Güvenc *et al*., 2012). The genus *Mindium* (*Michauxia*), a member of this family, is native to the Eastern Mediterranean, Turkey, the Caucasus, Iraq, and Iran. It is represented. by 7 species in the world; namely, *M. campanuloides* L'Hér., *M. koeieana* Rech., *M. laevigata* Vent., *M. nuda* DC., *M. stenophylla* Boiss. and Hausskn., *M. tchihatchewii* Fisch. and C.A.Mey., and *M. thyrsoidea* Boiss. and Heldr (Al-Zein *et al*., 2004), three of which are indigenous to Iran (Rechinger *et al*., 1965). *M. laevigata* is a dense bush with a characteristic aroma and small green leaves that is mainly distributed in central parts of Iran where it is locally known as ''Gole Shekafteh". Analysis of the volatile constituents of the *M. laevigata* oil has revealed the presence of thymol, alpha-terpinolene, caryophyllene oxide, and viridiflorol oils in it (Masoum *et al*., 2013). Moreover, the metanolic extract of the species has been recognized for its antibacterial and antifungal activities (Modaressi *et al*., 2013).

Identification of such biochemical features as antioxidant capacity of a medicinal plant is essential for its application. Reactive oxygen species (ROS) are produced in plants mainly under stress conditions; these may inflict damages to membrane lipids, carbohydrates, proteins, and nucleic acids, leading to stunted cell growth and development (Hader, 2002). Plant ability to counteract the adverse effects of environmental stresses to sustain its productivity is said be related to the scavenging capacity of the stress-induced toxic oxygen species (Salehi *et al*., 2012). Consumption of medicinal plants containing a high antioxidant capacity has been reported to provide a strong defense against ROS (Kanimozhi *et al*., 2011 and Mariutti *et al*., 2014).

There are differences, in most cases, among otherwise morphologically similar plant species growing in geographically different habitats (Sheidai *et al*., 2014). Problems associated with variability and plant growth conditions have led to great confusions in the precise identification of plants. This can be remedied, however, by recourse to molecular studies that help the exact identification of a plant species growing in different parts of the world (Zhang *et al*., 2014).

To be the best of the authors' knowledge, no pervious investigation hasyet been conducted into the antioxidant capacity and total protein content of *M. laevigata*. Neither has any data been provided on the molecular profile of the plant. The present study was, therefore, designed and implemented to evaluate the antioxidant enzymes and total protein content in the

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different parts of the species during its vegetative and generative stages. In addition, molecular studies were carried out to characterize the biosysteatic profile of *M. laevigata*.

Materials and Methods:

Plant material: Plant samples were collected from the various parts of *M. laevigata* growing in Gharedagh Mountains (N: 36°37'45.2''; E: 48°31'50.1'') in Zanjan. The taxonomic identification was accomplished by the Department of Biological Sciences at Zanjan University.

Enzyme assay: Fresh leaf samples were employed for enzyme analysis. For this purpose, leaves were frozen in liquid nitrogen immediately after harvesting and stored at –20 °C for enzyme assays. One gram of the leaf material was homogenized in 3 ml of 0.05 M Na-phosphate buffer (pH 7.8) containing 1 mM ethylene diamine tetra acetate (EDTA) and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged at 14,000 revolutions per minute (rpm) for 30 min at 4 °C and the supernatants were collected and used to determine enzyme activity. All the assays were accomplished at 4 °C and the spectrophotometric analyses were conducted on a Shimadzu (UV-1600) spectrophotometer.

Peroxidase (POD; EC 1.11.1.7) activity was based on the method described in Herzog and Fahimi (Herzog and Fahimi ,1987). According to this method, the increase in absorbance is measured at 465 nm for the formation of 0.15 M Na phosphate citrate and oxidized3,3′-Diaminobenzidine (DAB). One enzyme unit is defined as µmol ml^{-1} of H_2O_2 destroyed per minute.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada (Nakano and Asada ,1987). The assay depended on the decrease in absorbance at 290 nm during the oxidation of ascorbate. One enzyme unit is defined as µmol ml^{-1} of ascorbate oxidized per minute.

Catalase (CAT; EC 1.11.1.6) activity was determined according to the method described by Aebi. The reaction mixture contained the enzyme extract, 100 mM potassium phosphate buffer (pH 7.0), and 10 mL of 30% H_2O_2. The reaction was initiated by adding H_2O_2 and absorbance was measured at 240 nm. One unit of CAT was defined as the amount of enzyme to catalyze the decomposition of 1 mmol of H_2O_2 per minute.

Protein analysis: The quantitative analysis of protein at vegetative and generative phases were accomplished according to Bradford (Bradford ,1976) using bovine serum albumin as the standard. For qualitative protein analysis, the electrophoretic patterns were verified using 12% polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli ,1970) and the protein bands were stained with Coomassie Brilliant Blue R250. In addition, the quantitative and qualitative parameters of total protein were assessed using the TotalLab software.

Isolation of genomic DNA: Genomic deoxyribonucleic acid (gDNA) collected from various samples was extracted by DNAeasy Plant mini-kit (Qiagen) according to the manufacturer's instructions. The quality of gDNA was checked by electrophoresis on a 1% agarose gel.

Determination of DNA purity: The concentration and purity of gDNA were determined by measuring the absorption of ultraviolet light at 260 and 280 nm using a DR 5000 UV-Vis spectrophotometer (Hach).

Polymerase chain reaction: The 18S Ribosomal deoxyribonucleic acid (rDNA) sequence was extracted by polymerase chain reaction (PCR) from gDNA using the forward Internal Transcribed Spacer 1(ITS1) (5′ GGAAGGAGAAGTCGTAACAAGG 3′) and the reverse (ITS4) (5′ TCCTCCGCTTATTGATATGC 3′) primers. The PCR contained 2 µl forward primer (10 pmol), 2 µl reverse primer (10 pmol), 2 µl Magnesium chloride (MgCl2) (50 pmol), 5 µl PCR buffer (10x), 1 µl template (1µg), 1 µl Deoxynucleotide (dNTP) mix (10 mM), 1 µl Smart-Taq DNA polymerase (5U/µ), and sufficient autoclaved distilled water to reach a volume of 50 µl. PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min, a 35 cycle amplification (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min), and a final extension for 5 min at 72 °C. The PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Sequencing: The PCR product was cleaned up by AccuPrep^R PCR Purification Kit (Bioneer, Korea) before it was sequenced by an automatic sequencer (Bioneer). ITS1 and ITS4 were applied as universal primers.

BLAST and phylogenetic analyses: Similarity searches were carried out using BLASTN (Altschul *et al*., 1997) on the server at the National Center for Biotechnology Information (NCBI) server. Nucleotide sequences were derived from GenBank (Hermjakob *et al*., 2004) and SwissProt (Bairoch and Apweiler 1999) databases. The multiple sequence alignment was performed using the CLUSTALW (version 1.9) program and a phylogenetic tree was drawn using the neighbor-joining method in the NCBI server.

Statistical analysis: Each experiment was conducted in triplicates. The results are depicted as bar graphs and expressed as means ± SE as standard error. Statistical significance was evaluated by a one-way ANOVA followed by the Dennett's test.

Results and Discussion:

POD, APX and CAT assays: It was the main objective of the present work to detertmine the presence of antioxidant enzymes in *M. laevigata.* POD Activity was found in the extracts of the roots at both vegetative and generative phases (Fig. 1). Moreover, a significantly ($p \geq 0.05$) higher root POD activity was detected at the generative phases than at the vegetative ones while no significant POD activity was observed in other parts of

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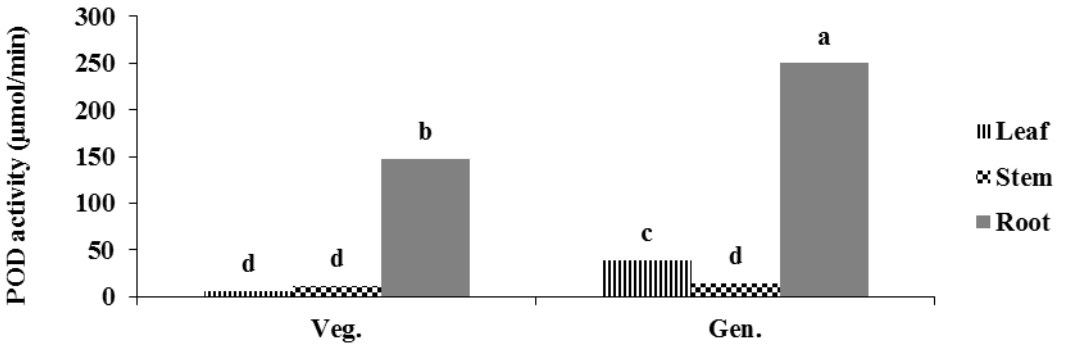


Figure 1. POD activity in the different parts of *M. laevigata* at both vegetative and generative phases. Vertical bars represent ± S.E. and the same letters are not significantly different at 5% level

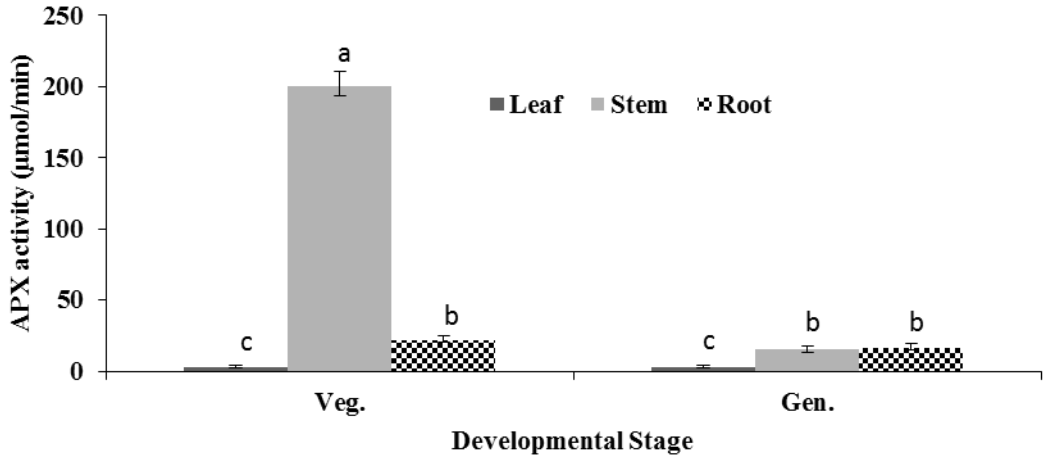


Figure 2. APX activity in the different parts of *M.laevigata* at both vegetative and generative phases. Vertical bars represent ± S.E. and the same letters are not significantly different at 5% level.

the plant at any of the developmental stages significant POD activity was observed in other parts of the plant at any of the developmental stages investigated. POD has been suggested to play a key role in stiffening cell walls through the formation of cross-links between cell wall polymers (Lin and Kao, 2001). Talano et al. reported that root cells express the POD gene to decrease the adverse effects of growth medium conditions. Our results revealed that the root of *M. laevigata* is the main source of POD production.

Contrary to POD, APX activity was detected at the vegetative phase. Its activity was significantly higher in stems than in all other parts of the plant (Fig. 2). During the generative stage, however, only negligible amounts of APX activity were observed in all the plant parts. APX has been reported to play a key role in the detoxification of ROS, especially for H_2O_2 removal from the cells (Hameed *et al.*, 2011). Moreover, cells containing adequate amounts of ascorbic acid have been shown to express APX during plant growth and development (Athar *et al.*, 2008). Finally, our results revealed no APX activity in the leaves, a finding that is in agreement with those of Pe´rez et al. who detected no APX activity in grape leaf extracts.

As shown in Fig. 3, CAT activity was observed in significant amounts only in the stems of *M. laevigata* at its generative stage. During the vegetative phase, however, only a slight amount of CAT activity was detected in the plant samples. Research has shown that CAT serves as an outstanding enzyme that scavenges H_2O_2 in peroxisomes. Liu et al. Maintained that CAT activity is the most effective antioxidant enzyme in preventing cellular damages.

Quantity and quality of total protein content: The protein contents in different parts of *M. laevigata* were investigated both quantitatively and qualitatively. The results of the quantitative investigations indicate that leaves had the highest amount of protein among the plant parts (Fig. 4). It was also found that the plant had greater protein contents in all its parts at the vegetative rather than at the generative stage. During the vegetative phase, plant growth increases progressively with increasing photosynthesis. Many new proteins involving tissue structure are synthesized in the leaf cells as the major sites in whose ribosomes proteins are synthesized (Arzani *et al.*, 2004). Proteins are being implicated in an expanding catalogue of physiological functions in plants. While certainproteins are required for plant responses to environmental conditions (Duman and Wisniewski, 2014), others are involved in such

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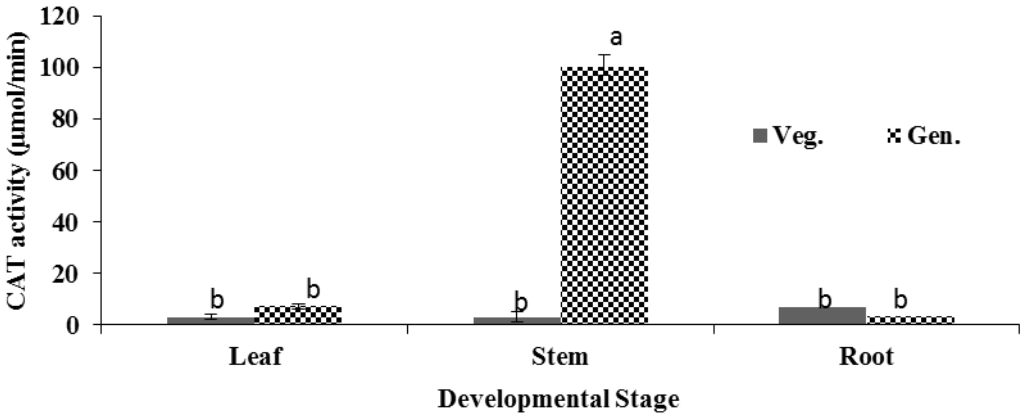


Figure 3. CAT activity in the different parts of *M.laevigata* at both vegetative and generative phases. Vertical bars represent ± S.E. and the same letters are not significantly different at 5% level.

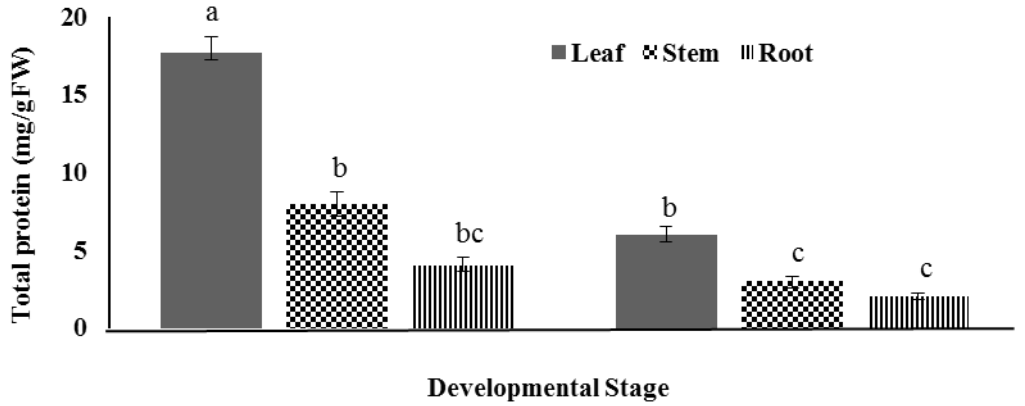


Figure 4. Total protein content in the different parts of *M.laevigata* at both vegetative and generative phases. Vertical bars represent ± S.E. and the same letters are not significantly different at 5% level.

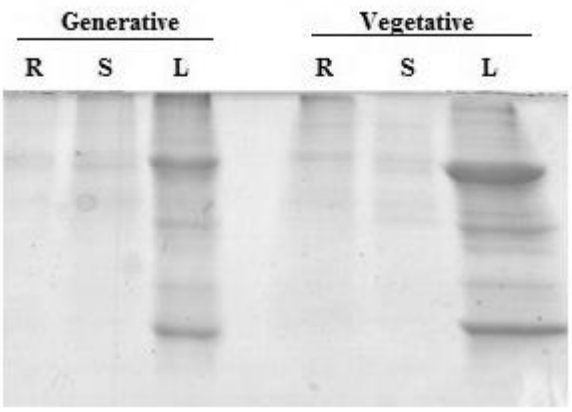


Figure 5. Protein profile analysis by SDS-PAGE at the different developmental (vegetative and generative) stages in the roots (R), stems (S), and leaves (L) of *M.laevigata*.

processes as catalyzing chemical reactions (enzymes), facilitating membrane transport, intracellular structure, and energy generating reactions involving electron transport (Jamet *et al.*, 2006 and Di Matteo *et al.*, 2003). Certain proteins have also been reported to be responsible for the turgidity and stability of membrane transport from leaves to stems (Ringli *et al.*, 2001). Finally, our results showed that stems contain higher amounts of protein than the roots do, which can be attributed to the greater metabolic activity of the stems than that of roots.

The qualitative investigations of the protein content in *M. laevigata* was accomplished using SDS-PAGE denaturation (Fig. 5). The protein bands depicted in the vegetative phase were found to be more intensive than those in the generative stage. In addition, the leaf protein bands were detected to outnumber those of the stem or the root. These qualitative data confirmed the validity of our protein quantitative results. Because of their metabolic activity, proteins are concentrated in the

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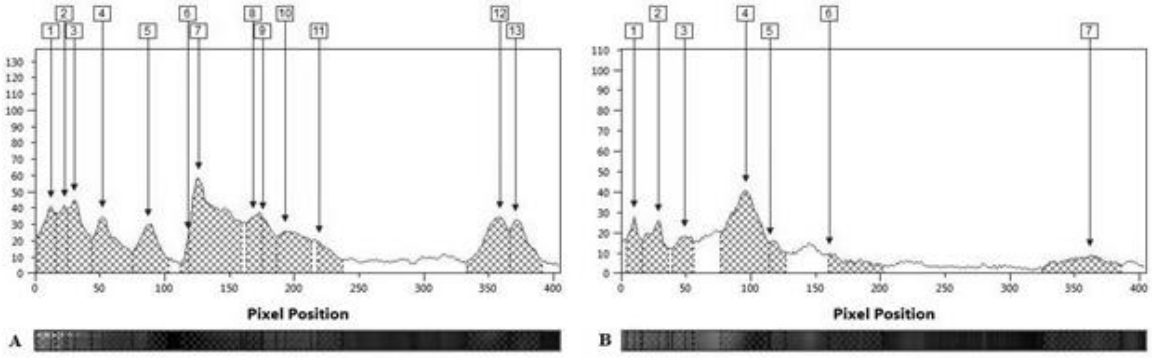


Figure 6. Densitometry analysis of total protein in leaves at the vegetative (A) and generative (B) phases. Digits represent the number of protein, and the area surrounded by each peak shows protein concentration.

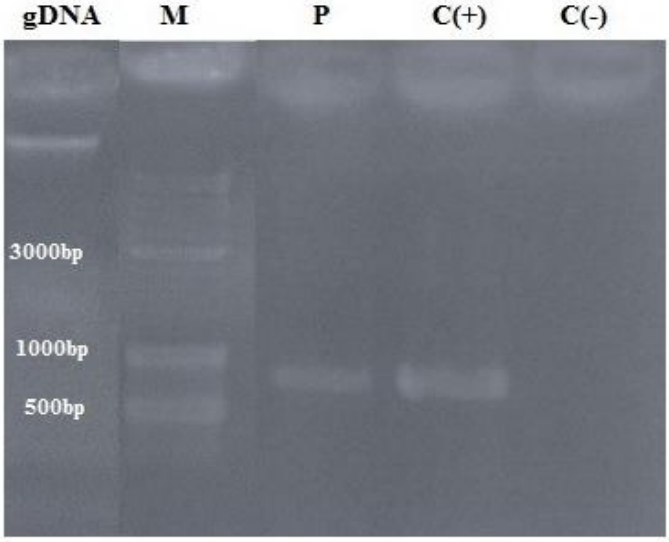


Figure 7. Agarose gel (1%) showing genomic DNA (gDNA) extraction and amplification of 18s rDNA from *M. laevigata*; M (DNA Marker), P (PCR product of amplification of 18s rDNA using ITS primers), C (+), and C (-); control positive and negative, respectively

leaves. Thus, it is likely that the presence of proteins can be exploited as indicators of the developmental changes in plant life.

The results of protein densitometry are shown in Fig. 6. Clearly, leaf proteins at the vegetative stage are higher in both numbers and concentrations than they are at the generative phase. This is evidenced by the 13 proteins detected during the vegetative phase against only 7 identified at the generative phase. The area surrounded by each peak in the vegetative stage in Fig. 6 is larger than that in the generative phase. This means that the total protein concentration in the vegetative stage is also higher than that in the generative stage. These results verify the quantitative and qualitative results obtained for the total protein content. In addition, the densitometry analyses of other plant parts confirmed the considerable differences already established between total protein content at both the developmental stages (data not shown).

DNA extraction, PCR, and sequencing: The authors' literature review showed that no previous study reported on the rDNA sequence of *M. laevigata*. In this study, the gDNA extraction and rDNA amplification are represented via ITS1 and ITS4 primers for the first time. The plant's gDNA was extracted by DNAeasy Plant mini-kit (Qiagen) while the PCR products were successfully cleaned up and sequenced as well. The sequencing results showed a 790 bp fragment (Fig. 7).

BLASTN and phylogenetic tree: The ITS sequence of the plant was also blasted against the NCBI database with maximum identity (greater than 97%) to determine the molecular differences between the plant and the previously reported sequenced species of the *Campanulaceae* family. The closest match in the database was recorded and sequenced. The phylogenetic tree drawn by NCBI is shown in Fig. 8. The methods developed for the study of plant biosystematics and diversity is quite recent (Wang *et al.* 2009). Some original plants of the *Campanulaceae* family, but not all, have been already listed on GenBank records. In our BLASTN results, *M. laevigata* was identified by Internal Transcribed Spacer (ITS). ITS primers have been reported to contain more different loci than chloroplast regions (Prebble and Cupido, 2011).

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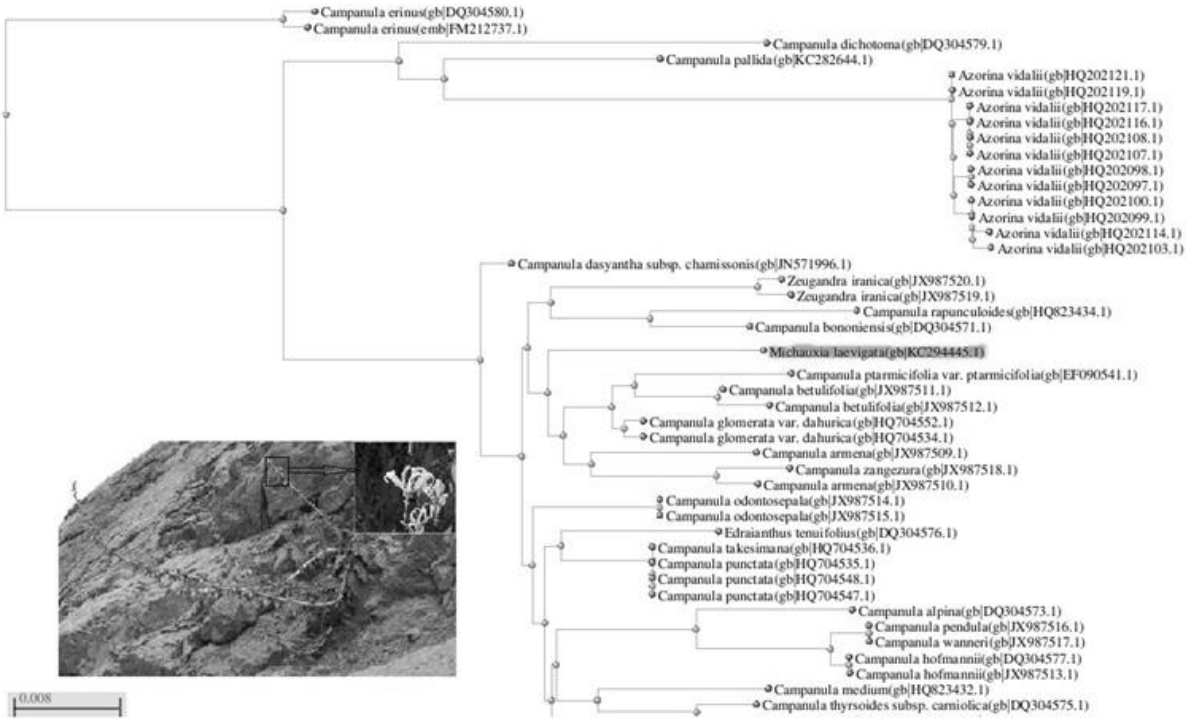


Figure 8. Phylogenetic tree of *M.laevigata* by the neighbor-joining method in the NCBI server; maximum sequential difference 0.1. The genus highlighted by yellow is the one detected in the present study and inscribed under the accession number KC294445.1

Mindium was identified at the genus level by BLASTN and grouped in a branch separated from other genera. According to the phylogenetic tree, the closest genus to *Mindium* based on identity percentage was *Campanulae glomerata* var. *dahurica*. In our future work, we intend to describe and characterize the DNA sequence of *Mindium* based on the knowledge gained so far. These molecular approaches help identify a large number of plant species which are required for the community biosystematic studies. Based on our molecular analyses, a novel gene was inscribed in the GenBank under the accession number KC294445.1. The isolation and identification of *Mindium* by means of molecular analysis associated with morphological techniques will be useful in upcoming investigations of plant ecology.

Conclusion:

The antioxidant capacity and protein content of the different organs of *M. laevigata* were successfully characterized for the first time in this study. Moreover, the DNA sequence of the plant was identified and reported, which might be of great importance in determining the genetic diversity in the genus *Mindium* at the species level. Based on the results obtained, it might be claimed that multiple parts of the plant are involved in the constitution of the antioxidant capacity of *M. laevigata*. Additional research is required to shed light on all the biochemical aspects of *M. laevigata*.

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