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Dissimilarity in the Nuclear Ribosomal DNA Internal Transcribed Spacer Regions of *Haplophyllum* spp. Founded in Ashdagh Mountain, Sangaw, Kurdistan of Iraq

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ABSTRACT

Studying and understanding the changes in the molecular sequences of plants are important to better identification and classifying the species. Internal transcribed spacer (ITS) regions are consider an informative genetic sequence to find out the variation among species of the same genus. In this study, ITS regions of *Haplophyllum* collected in Ashdagh Mountain were amplified, analyzed, and compared with reference species gathered from gene bank. The results showed that the collected species was closely related to *Haplophyllum blanchei* and *Haplophyllum tuberculatum*. In addition, there were differences in the number of the base pairs in the ITS1 region between *Haplophyllum* sp. and *H. blanchei* and *H. tuberculatum*. The transition to transversion ratio between *Haplophyllum* sp. and *H. tuberculatum* was lower (=1) than with other species. The results reveals that the studied plant is could be a new species in Iraq. To the best of our knowledge, this study is the first molecular taxonomic study done to identify *Haplophyllum* species in Ashdagh mountain in Sangaw/Suleimani.

Index Terms: ITS regions, Base pairs, Transversion, Molecular, Sangaw/Suleimani

1. INTRODUCTION

The genus *Haplophyllum* is consider one of the most common and rich genera in Rutaceae family [1,2]. The family includes 69 species of *Haplophyllum*. This genus is widely distributed throughout tropical, subtropical, and temperate regions [3,4]. Studies showed that some species of this genus contain flavonoid, lignans, alkaloids, and glycosides [5-7].

These phytochemical properties of the species made it an important medicinal plant [8]. The life form of the species is characterized as perennial herbs, growing on rocky hills, slopes, sandy soil, stony mountains, or steppes [2,3].

Many morphological studies have been conducted on the species of this genus and some new species have been identified recently. For example, new species (*Haplophyllum ermenekense*) has been identified as new species in Turkey [9]. However, few species have been identified or studied through molecular approaches [2]. Nuclear ribosomal internal transcribed spacer (ITS) regions are more powerful than other plastid regions in identification of intraspecific variability among species of the same genus [10-12]. ITS regions are characterized as ITS1 and ITS2. In addition, the high evolution rates of these two regions make them more useful for studying and trucking the diversification levels among closely related species [13]. Many new plant species have been identified and recorded based on ITS regions [14]. Sangaw area with unique

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geological and ecological characteristics (abiotic factors) would have a massive influence on the *Haplophyllum* species that would be assessed at the molecular level. We predict that the ITS regions will be different in compare with other available reference species in GenBank. The rational for our prediction is because the surveyed has its unique geological and ecological characteristics (abiotic factors) and it is possible that the abiotic factors have significant influence on the composition of ITS sequences. Therefore, the aim of this study is to examine the ITS regions of *Haplophyllum* collected in Ashdagh mountain in Sangaw. Sangaw is a district located in the south east of Sulaimani Province The aim of this study is to examine the ITS regions of *Haplophyllum* collected in Ashdagh mountain in Sangaw (a district located in the south east of Sulaimani Province).

2. MATERIALS AND METHODS

2.1. Sample Collection

The leaves samples (ten leaves) were collected from the unknown plant sample founded on Ashadah Mountain in Sangaw (Latitude: 35° 12' 49", longitude: 45° 33' 47") in February 5, 2021 (Fig. 1). The collected samples placed inside zipper plastic bags within ice box. The zipper bags were transferred immediately to lab and stored in deep freezer to prepare it for molecular analysis.

2.2. DNA Extraction and Polymerase Chain Reaction (PCR)

AddPrep Genomic DNA Extraction Kit was used to extract the total genomic DNA. Two universal nuclear regions named ITS 1 and 2 (ITS1 and ITS2) primers were used to identify the unknown species based on the phylogenetic analysis. The primes for ITS1 are ITS1F (ITS1 forward) (5'- GGAAGKARAAGTCGTAACAAGG -3') and ITS1R (ITS1 reverse) (5'- GCGTTCAAAGAYTCGATGRTTC-3') and primers of the ITS2 gene are ITS2F (ITS2 forward) (5'- CAWCGATGAAGAACGYAGC-3') and ITS2R (ITS2 reverse) (5'-RGTTTCTTTTCCTCCGCTTA-3') [15]. In the standard PCR, each reaction contained (MyTaqTM HS Mix-Bioline, USA) master mix (10 μL) 10 pmol of each primer and 20 ng template DNA in a final volume of 20μL. PCR was performed using a three-step cycling protocol: initial denaturation (95°C/5 min) 1 cycle; ([denaturation (95°C/30 s), annealing (57°C/30 sec), (extension 72°C/30 s) 40 cycles]); and final extension (72°C/5) 1 cycle (Bio-Rad C1000 Thermal Cycler, USA). The PCR products were analyzed in 1% agarose gel (Only 1 g of agarose was dissolved in 100 mL (1X TAE buffer) TAE: Tris-acetate EDTA) stained with Ethidium bromide (0.07% ready to use). PCR products were purified and sequenced, the sequencing reactions were investigated for both strands of all two purified PCR products using HiSeq4000 (Illumina, San Diego, USA) of Macrogen Inc., Korea.

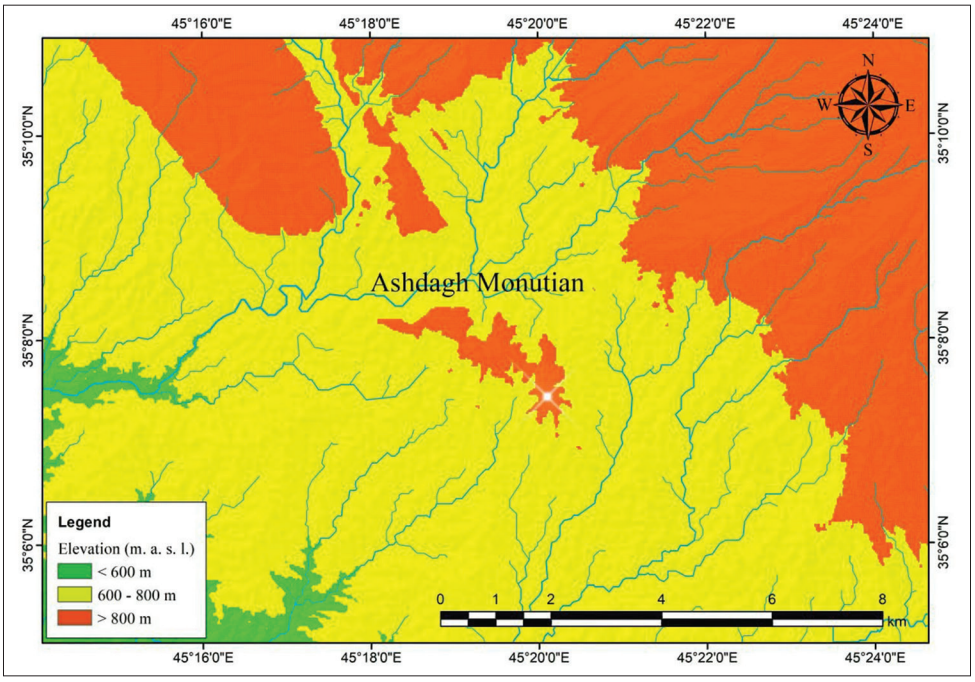

Fig. 1. Location map of study area. The white dot indicates the collection site.

The forward and reverse sequences of ITS1 and ITS2 of plant sample were combined with each other using Bioedit software v.7. [16] Blast software in National Center for Biotechnology Information (NCBI) database was used to search the identity of our sequence. Then, the species with high percentage identity (Table 1) were downloaded from GenBank (NCBI) and aligned using ClustalW embedded in Bioedit.

2.3. Sequence Analyses and Phylogenetic Tree

The pairwise distance (i.e. divergence) among *Haplophyllum* species was estimated using MegaX. Tamura 3-parameter (T92 + G) was picked as a best model to estimate the distance. Furthermore, pairwise deletion was used to treat the gaps among the sequences. PAUP 4.0 [17] was used to estimate transition and transversion rates (Table 2). The phylogenetic tree (Fig. 2) was reconstructed using Maximum likelihood (ML). MegaX was used to generate the trees and T92 with gamma distribution (+G) was picked as best substitution model. To test the confidence of the clades, bootstrap (BS) method was performed with 100 replicates.

3. RESULTS AND DISCUSSION

In comparison with reference species, the unknown sample showed that the plant genus is belong to *Haplophyllum* and the percent identity of *Haplophyllum* sp. (samples) was 93.4% with *Haplophyllum tuberculatum* and 92.34% with *Haplophyllum blanchei* (Table 1).

The analysis of the sequences (Table 2) showed that the proportion of the difference (i.e., pairwise distance) among *Haplophyllum* sp., *H. tuberculatum,* and *H. blanchei* is 2% and it is less than others. In addition, the ratio of base pairs transition to transversion between *Haplophyllum* sp. and *H. tuberculatum* is 1% which means that the rate to transition (CT = 4) is equal to transversion (AT = 1, CG = 2, and GT = 1). On the other hand, *H. blanchei* had high transition to transversion rate (ratio = 3) in comparison with other species. The alignment of the sequences illustrates a clear difference in ITS1 region in *Haplophyllum* sp.

In compare with other species sequences (Fig. 3). For example, there is a clear difference in nucleotide composition among *Haplophyllum* sp. and *H. tuberculatum* and *H. blanchei* in position 14 to 98 (Fig. 2). *Haplophyllum* sp. has 84 and 15 more nucleotides than *H. tuberculatum* and *H. blanchei,* respectively. The length of sequence in *Haplophyllum* sp. was 589 base pairs which is longer than other species.

The ML tree strongly support the clade of *Haplophyllum* sp. and *H. tuberculatum* and *H. blanchei* (BS = 100) (Fig. 2). Based on the

TABLE 1: The percentage identification (Per. Ident) of the plant sample (*Haplophyllum* sp.) with available reference species in GenBank

No.	Scientific Name	Max Score	Total Score	Query Cover	Per. ident	Acc. Len	Accession
1	*Haplophyllum tuberculatum*	739	883	73%	93.4	635	KF805116.1
2	*Haplophyllum blanchei*	730	868	74%	92.32	637	AY484571.1
3	*Haplophyllum linifolium*	656	656	62%	90.64	628	AY484572.1
4	*Haplophyllum coronatum*	647	647	62%	90.25	630	AY484573.1
5	*Haplophyllum bastetanum*	645	645	62%	90.25	627	AY484576.1
6	*Haplophyllum rosmarinifolium*	638	638	62%	90.06	626	AY484574.1
7	*Haplophyllum suaveolens*	558	558	62%	86.77	625	AY484575.1
8	*Ruta montana*	283	283	50%	80.09	612	AY484577.1

TABLE 2: Sequence analysis of *Haplophyllum sp.* and other reference species from GenBank database (i.e., NCBI; National Center for Biotechnology Information)

No	Taxa	Ti		Tv				Ident				Prop diff	Ti/Tv ratio	Total
	Haplophyllum sp. vs. :	AG	CT	AC	AT	CG	GT	AA	CC	GG	TT			
1	*Haplophyllum tuberculatum*	0	4	0	1	2	1	87	176	152	79	0.02	1	502
2	*Haplophyllum blanchei*	2	7	0	0	3	0	98	205	173	84	0.02	3	572
3	*Haplophyllum linifolium*	4	8	1	1	4	3	80	164	144	77	0.04	1.33	486
4	*Haplophyllum coronatum*	5	9	4	2	3	2	82	162	143	76	0.05	1.27	488
5	*Haplophyllum bastetanum*	4	9	1	1	4	3	79	164	144	76	0.05	1.44	485
6	*Haplophyllum rosmarinifolium*	5	8	1	1	4	3	79	163	143	77	0.05	1.44	484
7	*Haplophyllum suaveolens*	9	14	3	2	5	3	77	164	140	78	0.07	1.77	495
8	*R. montana*	5	16	5	3	6	4	67	114	110	63	0.1	1.17	393

The table include transition (*ti*) and transversion (*tv*) rates, rates of identical base pairs (*Ident*), proportion of difference (*Prop diff*), transition to transversion ratio (*ti/tv ratio*), and total length of the sequences (*total*)

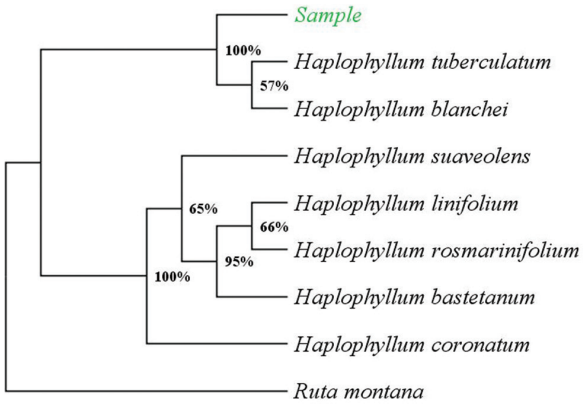

Fig. 2. Maximum Likelihood (ML) tree. The highlighted "sample" is the founded *Haplophyllum* sp. Numbers represent bootstrap values.

phylogenetic tree, *Haplophyllum* sp. is sister to subclade of *H. tuberculatum* and *H. blanchei*. ITS 1 and ITS2 are widely used in plant phylogenetic studies due to their ability to teasing out the intra- and interspecific variation among species [10]. In general, the base substitutions in the ITS region among *Haplophyllum* species are more than other species in angiosperm [2]. The high substitution rates among *Haplophyllum* species increase in their evolution rates [2,18,19]. The estimated transition/transversion bias (*R*) is 1.59. Substitution pattern and rates were estimated under the Kimura (1980) 2-parameter model [20]. The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -1322.866.

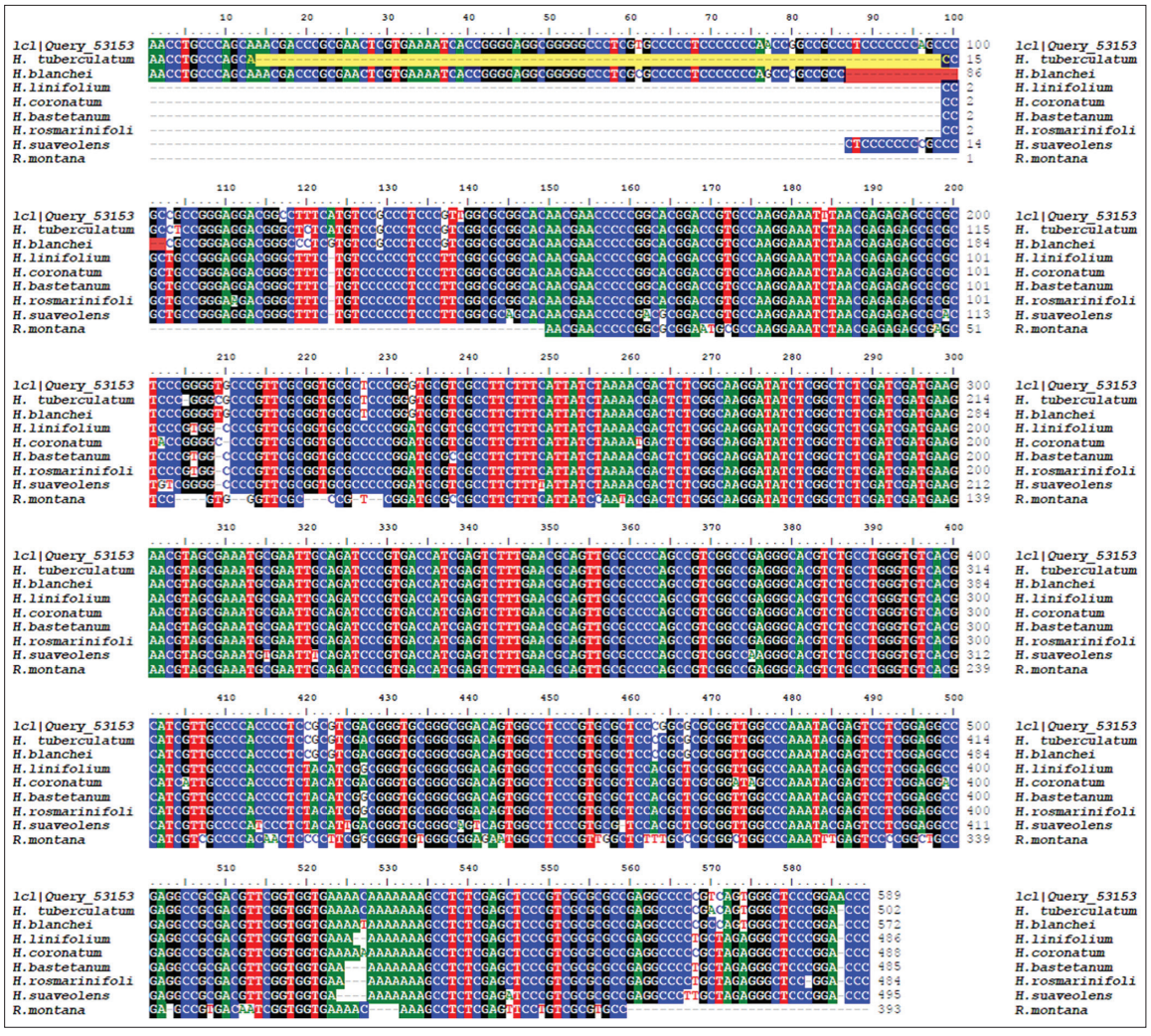

Fig. 3. Clustal W multiple sequence alignment of ITS1 and ITS2 regions. The sample species is indicated as "lcl|Query_53153" with 589 bp. The highlighted gaps with yellow and dark orange colors indicate the base pair differences among our sample species and *Haplophyllum tuberculatum* and *Haplophyllum blanchei,* respectively.

The analysis involved nine nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 589 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [21]

Furthermore, *H. tuberculatum* and *H. blanchei* are most abundant species in Kurdistan [22]. The phylogenetic and sequence analyses reveal that the founded species is closely related to the *H. tuberculatum* and *H. blanchei* and distantly related with others. The differences in the number of base pairs in the ITS1 region between *Haplophyllum* sp. and *H. tuberculatum* and *H. blanchei* are the main cause of the phylogenetic placement of the species in the clade. Based on the sequence information for the ITS regions in the GenBank, the finding species could be considered as a new species in Iraq and have not been submitted before.

4. CONCLUSION

The existed dissimilarities in the ITS1 sequence and phylogenetic position of *Haplophyllum* sp. in compare with other reference species reveals that the founded species is a different species. In addition, this study showed the importance of using molecular studies to see the variability among closely related species.

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