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Secondary Structure Form of *ITS2* Region: A Significant Labeling Tool at all Taxonomic Levels

Mevlüde Alev ATEŞ[1*], Pelin ACAR[2], Seher KARAMAN[3], Dudu Özlem MAVİ İDMAN[2], Selçuk Tuğrul KÖRÜKLÜ[4], Barış BANİ[5]

[1] *Kırşehir Ahi Evran University, Faculty of Agriculture, Department of Agricultural Biotechnology, Kırşehir, Türkiye.*
[2] *National Botanical Garden of Türkiye, Department of Collection, Ankara, Türkiye.*
[3] *Aksaray University, Faculty of Arts and Science, Department of Biology, Aksaray, Türkiye.*
[4] *Ankara University, Faculty of Science, Department of Biology, Ankara, Türkiye.*
[5] *Kastamonu University, Faculty of Arts and Science, Department of Biology, Kastamonu, Türkiye.*

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*** Corresponding Author**
E-mail: malev.ates@ahievran.edu.tr
https://orcid.org/0000-0002-2141-5438
https://orcid.org/0000-0001-8383-9431
https://orcid.org/0000-0003-1239-8266
https://orcid.org/0000-0002-3662-8970
https://orcid.org/0000-0002-7440-6818
https://orcid.org/0000-0001-5694-3700

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Abstract

The general purpose of the molecular systematic studies is to illuminate the *ITS2* structure of the target populations, to determine its phylogenetic boundaries, and to clarify intra-species and inter-species relationships. Particularly, the internal transcribed spacer 2 (*ITS2*) region of nuclear ribosomal DNA is used in molecular systematics because of availability of conserved regions with its highly repeated in number in plant genomes. Addition to the primary sequences of *ITS2*, also secondary structure form of the region became a valuable feature in species divergence and became to use like a morphological character. In the current study to indicate the secondary structure form of the *ITS2* region as a useful tool in systematics, different taxa from 22 genera were used. The DNA samples were collected in the field studies in 2021 and sequences were aligned using ClustalW and Kimura-2 parameter to calculate the genetic distances. Phylogenetic tree was also constructed with Maximum Likelihood method with the best suitable model at MEGA X software. The secondary structure predictions of species and ΔG (Gibbs) free energy calculations, the tools of both the *ITS2* database and mFOLD web server were used. The results indicated that *ITS2* secondary structure estimations represented the genetic differences visibly with its helices and motifs like a morphological character. Consequently, even if primary structure of the *ITS2* region revealed as valuable marker in molecular systematic studies, also, all tested secondary structure forms of the region will be used as an ideal marker for taxonomic and phylogenetic reconstructions at all taxonomic levels.

INTRODUCTION

DNA barcoding techniques are the most popular methods for plant sciences in recent years for species identifications (CBOL Plant working group, 2009) due to its non affectiveness property by external factors or development stage. Additionally, the main material of the DNA method can be easily isolated from all tissues (Sucher and Carles, 2008; Seethapathy *et al.*, 2015; Wu *et al.*, 2015; Mishra *et al.*, 2016). Hence this provides a powerful method for species identifications at all levels (Yu *et al.*, 2017). The important advantage of barcoding techniques is the usage of short sequences of standart part of the genome with respect to whole genome to detect the identity of samples. Especially rbcl and matK plastid coding genes which are the parts of chloroplast genome have been adviced as the most useful barcodes due to their ability at amplification and sequencing processes by CBOL Plant Working Group (2009) (Michel *et al.*, 2016; Mohamed, 2016; Al-Juhani and Khalik, 2021).

In recent plant phylogenetic studies, the internal transcribed spacer (*ITS*) region of rDNA is used as complementary for matK and rbcl regions with their relatively strong discrimination property (CPBG China Plant BOL Group, 2011). *NrDNA ITS* region is composed of 2 different intergenic regions with highly conserved 5.8

rRNA between them (Zhang *et al*., 2015). The *ITS* regions are not incorporated into mature ribosomes but also sustain an extra cleavage during the maturations of ribosomal RNAs that is assembled by the secondary structure of *ITS* (Edger *et al*., 2004). The *ITS2* sub region has also been explained as a valuable marker for identifying species at all levels (Chen *et al*., 2010; Yao *et al*., 2010; Han *et al*., 2013). Morover, the *ITS2* is shorter and easily amplified through *ITS1* and this property makes this region as a valuable marker in DNA barcoding techniques (Chen *et al*., 2010; Li *et al*., 2011). Both sequences of the region have clear characteristics that are described as being universal among eukaryotes (Mai and Coleman, 1997). Therefore, these specific features provide clearness in the alignments to *ITS*′ molecule for phylogenetic reconstructions (Caisova *et al*., 2013).

The RNA activity of cell is progressed by *ITS2* secondary structure form. Even if the different combinations of sequences are indicated in nucleotides, generally secondary structure of eucaryotics *ITS2* regions has 4 helices and common motifs (Coleman, 2007). Because of these conserved motifs, secondary structure forms of the *ITS2* region solve all problems and obtain more reliable perspective to relationships at higher taxonomic levels (Zahng *et al*., 2015). Addition to these properties, this secondary structure form is also controlled by basepair interactions between canonical base-pairs, non-canonical stable, unstable and uncommon pairs (Leontis and Westhof, 2001). Hence, these paired and unpaired *ITS2* structural states have special phylogenetic information, that is not found in the primary sequenceIn other words, this data can advance phylogenetic predictions (Telford *et al*., 2005).

In the current study, the purposes are;

1. To find out the answer of the question that *ITS2* is a valuable molecular marker for plants.
2. To emphasize the importance of secondary structure of *ITS2* region which is valuable in species divergence and used as a molecular morphological character.
3. To investigate differences between secondary *ITS2* structure form of different taxa and differences between taxa about Gibbs free energy values of *ITS2* secondary structure form related with conserved helices and motifs.

To do all above, different taxa from different genera were selected for understanding the phylogenetic relationships and to compare species divergence with both primary phylogenetic analysis and the secondary structure forms of *ITS2*.

MATERIALS and METHODS

In the current study, one of the aims is to show the significant morphological differences of *ITS2* gene regions by sampling different taxa of different genera from their varied habitats rather than to make systematic revision with the representative samples of the related families. Therefore, the fresh leaves of the 22 samples from 21 family (Table 1), which were choosen as representative species of its own family, were used to extract total genomic DNA via DNeasy Qiagen Plant Kit. For amplifiying *ITS* region, the primers pairs of the Hsiao *et al*. (1995) were used with a total volume of 25 µl composed of 4 µl 5 × Hot FirePol Blend PCR Mix (Solis Biodyne) (15mM MgCl2), 0.5 µl each primer pairs, 1.5 µl template DNA and 18,5 µl water. A thermo cycler (MultiGENE, Cleaver Scientific Ltd) was used for amplifiying the regions with the followings: 5 minutes at 95°C for initial denaturation, followed by 30 cycles of 30 seconds at 95°C for template denaturation, 30 seconds for annealing, and 90 seconds at 72°C for extension and 10 minutes at 72°C for final extensions. 2% agarose gel in electrophoresis were used to checked and all purifications and sequencing of products were done by BM Labosis Company (Ankara, Türkiye). Finch Tv software Version 1.4.0-manufactured by Geospiza Research Team (Patterson *et al.,* 2004) were used to check data after sequencing. MEGA (Molecular Evolutionary Genetics Analysis) 7.0.9 software (Kumar *et al*., 2016) was used with MUSCLE (Multiple Sequence Comparison by Log Expectation) tool (Edgar, 2004) for aligning the sequences. Aditionally, Neighbour Joining method with bootstrap test analysis with 1000 replicates was used to indicate an evolutionary perspective.

Additton to primary sequence analysis, secondary structures of *ITS2* sequences and ΔG (Gibbs) free energy calculations were predicted and calculated with the help of mFOLD web server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). The server was used at 37°C using RNA version 2.3 default parameters by the program parameters (Santa Lucia, 1998; Zuker, 2003).

Table 1. Studied species in the current study.

Species	Family	Collectors	Date	Location
Acorus calamus	Acoraceae	O.Mavi 1262, S.Karaman, T.Körüklü	29.07.2021	Yeniçağa Gölü / Bolu
Alisma plantago-aquatica	Alismataceae	O.Mavi 1258, S.Karaman, T.Körüklü	29.07.2021	Yeniçağa Gölü / Bolu
Bunium ferulaceum	Apiaceae	SK 4040	22.06.2021	Güzelyurt / Aksaray
Brassica elongata	Brassicaceae	SK 4041	08.06.2021	Güzelyurt / Aksaray
Arenaria macrosepala	Caryophyllaceae	SK 4024	08.06.2021	Ekecik / Aksaray
Ceratophyllum demersum	Ceratophllaceae	O.Mavi 1229, P.Acar, T.Körüklü	04.06.2021	Çubuk Karagöl / Ankara
Ecballium elaterium	Cucurbitaceae	SK 4036	20.06.2021	Helvadere / Aksaray
Dioscorea communis	Dioscoreceae	O.Mavi 1271, S.Karaman, T.Körüklü	30.07.2021	Abant Gölü / Bolu
Astragalus lycius	Fabaceae	O.Mavi 1163	17.05.2021	Çankaya / Ankara
Astragalus gummifer	Fabaceae	SK4044	24.06.2021	Alpu / Elazığ
Frankenia hirsuta	Frankeniceae	SK 4045	26.06.2021	Tuzgölü / Aksaray
Geranium tuberosum	Geraniaceae	SK 4012	03.06.2021	Akhisar / Aksaray
Magnolia grandiflora	Magnoliadeae	SK 4031	18.06.2021	NGBB / İstanbul
Nymphaea alba	Nymphaeae	O.Mavi 1255, S.Karaman, T.Körüklü	29.07.2021	Hamzabey / Bolu
Papaver rhoaes	Papaveraceae	SK4029	08.06.2021	Güzelyurt / Aksaray
Poa bulbosa	Poaceae	O.Mavi 1183, B.Bani	29.05.2021	Kalınkaya Köyü, Alaca / Çorum
Potamogeton natans	Potamogetaceae	O.Mavi 1263, S.Karaman, T.Körüklü	29.07.2021	Abant Gölü / Bolu
Rosa canina	Rosaceae	SK 4022	08.06.2021	Güzelyurt / Aksaray
Cruciata taurica	Rubiaceae	SK 4014	08.06.2021	Güzelyurt / Aksaray
Salix alba	Salicaceae	SK 4021	08.06.2021	Güzelyurt / Aksaray
Acer campestre	Spindaceae	O.Mavi 1234, P.Acar, T.Körüklü	04.06.2021	Çubuk Karagöl / Ankara
Verbascum cheiranthifolium	Scrophulaceae	O.Mavi 1272, S.Karaman, T.Körüklü	30.07.2021	Abant Gölü / Bolu

RESULTS and DISCUSSION

In the study even if there were different taxa from different genera, the *ITS2* region were obtained approximately 230 bp in length. Although variable sites were very high as expected, the singleton site number was 32 and GC% of the sequences was found as 52. For understanding the general phylogenetic relationships between taxa, the phylogenetic tree was constructed. As expected, there were high bootstrap values in the branches and many of them were mainly related with their morphologies. Besides, based on branch combinations, genetic differences were clear (Figure 1).

Improving the accuracy of the informative data, secondary structure form of the species were drawn. Generally, all Viridiplanteae species with4-helicodial ring model were seen by using the predictions (Figure 2). Additionally, the secondary structure forms indicated the major differences between taxa that were seen in the phylogenetic tree at the different branch with high bootstrap values. The output topology in the current study was coherent with the morphological/physiological identifications of the taxa. Therefore, secondary structure prediction of *ITS2* region contributed a well resolution for the discrimination of taxa. Genetically similar taxa formed not same but similar secondary structure and distinct taxa were indicated by distinctive shapes. Therefore, these estimations were reflected the differences like a morphological character with different helices shape (Figure 2).

In addition to related with these estimations, the mFold web server also calculated ΔG (Gibbs free energy) values (Table 2). The calculation of ΔG (Gibbs) free energy values with mFold program parameters for studied taxa based on the helices and angles in the secondary structure with Thermodynamic calculations (Santa Lucia, 1998) were reflected different values. So, even if the primary structure reflected a perspective for the differentiations between different taxa, also the secondary structure form of *ITS2* region and ΔG (Gibbs) free

Figure 1. Phylogenetic reconstructions based on *ITS2* region. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. All positions with less than 95% site coverage were eliminated.

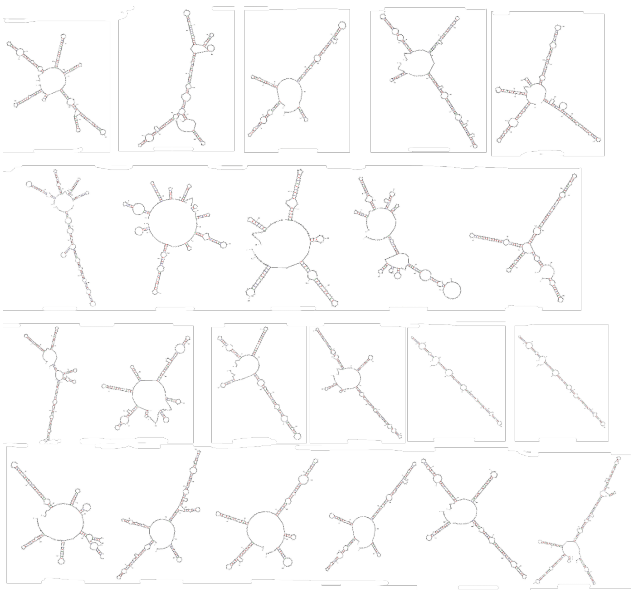

Figure 2. Secondary structure predictions of studied taxa. First line from left; *Cerataphyllum demersum, Geranium tuberosum, Acer campestre, Potomogedon natans, Cruciata taurica;* secondline from left; *Acarus calamus, Frenkenia hirsuta, Diosera communis, Verbascum cheiranthifolium, Poa bulbosa*; third line from left: *Magnolia grandiflora, Arenia macrosepala, Brassica elongata, Papaver rhoaes, Astragalus gummifer, Astragalus lycius*; last line from left: *Bunium ferulaceum, Ecballium elaterium, Salix alba, Rosa canina, Nymphaea alba*.

Table 2. ΔG (Gibbs) free energy values with mFold program parameters.

Species	**Family**	**ΔG energy(kcal/mol)**
Acorus calamus	Acoraceae	-72.0
Alisma plantago-aquatica	Alismataceae	-118.5
Bunium ferulaceum	Apiaceae	-72.6
Brassica elongata	Brassicaceae	-67.2
Arenaria macrosepala	Caryophyllaceae	-68.3
Ceratophyllum demersum	Ceratophllaceae	-105.6
Ecballium elaterium	Cucurbitaceae	-89.0
Dioscorea communis	Dioscoreceae	-49.0
Astragalus lycius	Fabaceae	-75.4
Astragalus gummifer	Fabaceae	-71.3
Frankenia hirsuta	Frankeniceae	-73.7
Geranium tuberosum	Geraniaceae	-82.3
Magnolia grandiflora	Magnoliadeae	-87.6
Nymphaea alba	Nymphaeae	-84.0
Papaver rhoaes	Papaveraceae	-81.9
Poa bulbosa	Poaceae	-90.4
Potamogeton natans	Potamogetaceae	-78.5
Rosa canina	Rosaceae	-72.2
Cruciata taurica	Rubiaceae	-89.8
Salix alba	Salicaceae	-92.1
Acer campestre	Spindaceae	-72.0
Verbascum cheiranthifolium	Scrophulaceae	-66.2

energy values were shown distinctions like a morphological characteristic of species due to its clear visuality between nucleotide sequences.

CONCLUSION

In recent phylogenetic studies, it was revealed that only one marker could not support the species discriminations. Especially, in DNA barcoding studies, using 2 or more markers could eliminate misunderstandings in phylogenetic estimations. Therefore, incongruence among gene phylogenies using multiple evolving markers could be highly useful (Edwards, 2009; Koch *et al*., 2007). Multi DNA barcoding is promising as the global standard of species identification, but it requires an agreement about barcode region, which is useful for all or selected taxa. At present, there is no standard DNA barcode region and also there are many attempts made so far. In this study, 22 accessions belong 21 family were used to evaluate discriminative potential of the secondary structure of *ITS2*. Especially, such DNA regions in field plants at high taxonomic levels has been signed as a valuable application if their polymorphism rates are high. It prevents time and money consumption while giving clarity at species level identification (Newmaster *et al*., 2006). It is concluded that choosing a single and short marker with high polymorphism rate could give advantages in the studies. Furthermore, the secondary structure of *ITS2* consists of a number of paired regions like multi-loci (Zhang *et al*., 2015). Also, it is firmly revealed the ΔG energy of secondary structure that gave a difference value based on the angles between helices of the taxa. Therefore, the effectiveness of *ITS2* barcoding in detecting, identifying, and classifying the genetic relationships between taxa make the region valuable in recent studies even if it is a short-region DNA barcodes and had a strong discriminatory power. It is believed that, using *ITS2* as a barcode region will be a valuable tool to validate the identification of taxa at all taxonomic levels with both primary and secondary structure estimations.

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ADDITIONAL INFORMATION

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