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# The Structural Features and Phylogenetıc Utiliti of the ITS in *Ferulago* W. Koch (Umbelliferae) Genus

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**Abstract:** To identify important structural features in the intergenic sequences of ribosomal DNAs, the nucleotide sequence of the 18-28S rRNA intergene region was determined in *Ferulago* genus. During the pre-rRNA processing pathway the corrected and efficient excision of the internal transcribed spacers (ITS1 and ITS2) is a crucial step for the biogenesis of a active ribosomal subunits. The recognition elements located in ITS were conserved among *Ferulago* species. While a striking sequence homology was observed in the mature ribosomal sequences, the two internal transcribed spacer regions were found to be G+C rich. Some variations among ITS sequences of this genus is found among *Ferulago* species.

**Key words:** *Ferulago*, ITS1, ITS2, rDNA, RNA processing, polymorphism

## INTRODUCTION

Apiacea (Umbelliferae) is a well-known family of flowering plants that includes many economically important plants (e. g., carrot, parnsnips, parsley, celery, fennel, dill, anise, cumin). Characteristic inflorescences (typically in umbels) and fruits of Apiaceae as well as their distinctive secondary compounds reflected in odors, flavors an deven toxicity, maket his family stand out among other angiosperms (Davis, 1972).

*Ferulago* W. Koch. (Umbelliferae) centered in Anatolia. This genus is represented by thirty two species in Turkey, 15 of these are only endemic to Turkey (Saya, 1985; Akalın, 1999).

The ITS region is part of the transcriptional unit of nrDNA, but the spacer segments of the transcript are not incorporated into mature ribosomes. ITS1 and ITS2 regions of the nrDNA transcript function in the maturation of nrRNA (Joseph *et al*., 1999; Oliverio *et al*., 2002). Useful variation must be more highly concentrated with in a set of ITS1 and ITS2 sequences than in longer DNA regions. Nucleotide differences corresponding to a variable region should be considered as resulting from a single mutation event (i.e., Block replacement by recombination). Recombination is responsible for the exchange of nucleotides blocks. Recombination between rDNA loci can lead to the shuffling of nucleotide blocs and can either maintain variability.

The nuclear ribosomal RNA genes (rDNA) of higher plants are organized in long tandem repeating units (Baldwin *et al*., 1995).

Higher order structure in the pre-ribosomal particle may be necessary to organize the processing sites in close spatial proximity (Tague and Gerbi, 1984). Small hairpin structures located near the processing sites may be involved in the maturation of the pre-rRNA The recognition signals for the processing enzymes lie more in the higher order structure than in the primary structure (Nazar *et al*., 1987; Wenner *et al*., 2002).

Eleven essential proteins constitute a highly conserved eukaryotic RNA processing complex called an exosome containing multiple exoribonuclease activities (Serin *et al*., 1996). Cis-acting elements and numerous essential trans-acting factors including endo and exoribonucleases and several snoRNA are required for spacer excision (Liang and Fournier, 1997; Sharma and Tollervey, 1999).

Although the comparative approach has been very useful in studies on the structure of mature ribosomal RNAs, thus, comparative studies of the spacer regions have not resulted in a universal model for their structure or processing (Doyle *et al*., 1994; Alvarez and Wendel, 2003). While the mature RNAs are very similar in size and nucleotide sequence, the internal transcribed spacer regions (ITS1 and ITS2) have found to differ greatly with respect to length and show very little sequence homology. We have been examining the conserved sequences of ITS1 and ITS1 from *Ferulago* species.

## MATERIALS AND METHODS

**Plant material collection:** Fruits of ten *Ferulago* species collected from Western Turkey were indicated

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Table 1: Location of *Ferulago* species collected for ITS1 and ITS2 sequence analysis and accession numbers of these sequences

| Species | Locality and collector | GenBank accession numbers |
|---|---|---|
| *F. galbanifera* Miller | Çanakkale, Çan-Yenice, N. and E. Özhatay, E. Akalın | ITS1: AJ972385; ITS2: AJ972386 |
| *F. asparagifolia* Boiss | İzmir, Selçuk Efes ruins, E. Akalın, U. Uruşak | ITS1:AJ972387; ITS2: AJ972388 |
| *F. thirkeana* Boiss | İstanbul, Çatalca, Ş. and T. Kültür | ITS1:AJ972391; ITS2: AJ972392 |
| *F. silaifolia* Boiss | Bursa, İnegöl, Mezitler II valley, N. and E. Özhatay, E. Akalın | ITS1:AJ972389; ITS2: AJ972390 |
| *F. trachycarpa* Boiss | Edremit, Zeytinli-Yaylatepe, road side, Ş. Kültür, E. Akalın | ITS1:AJ972393; ITS2: AJ972394 |
| *F. humilis* Boiss | Çanakkale-Kaz Mountain, N. and E. Özhatay, E. Akalın | ITS1: AJ972901; ITS2: AJ972902 |
| *F. sandrasica* Peşmen | Muğla, Köyceğiz, Sandras Mountain, N. Özhatay, E. Akalın | ITS1: AJ972897; ITS2: AJ972898 |
| *F. macrosciadia* Boiss | Çanakkale, Çan-Yenice, N. and E. Özhatay, E. Akalın | ITS1:AJ972903; ITS2: AJ972904 |
| *F. mughlae* Peşmen | Muğla, Köyceğiz, Kersele stream, E. Akalın, U. Uruşak | ITS1: AJ972899; ITS2: AJ972900 |
| *F. confusa* Velen | Tekirdağ, Hayrabolu, Şalgamlı village, N. and E. Özhatay, E. Akalın | ITS1:AJ972895; ITS2: AJ972896 |

Table 1. These plant materials kept -20°C until we examine (Table 1).

**DNA isolation:** DNA isolation and other procedures were achieved at Molecular Biology Laboratory of Hacettepe University, Ankara-Turkey between 2003 and 2004 years. Total genomic DNA were isolated from endosperm layer removed from dried seeds (eight seeds of each individuals) of ten *Ferulago* species (McDonald *et al*., 1994)

**Amplification of ITS region:** Double stranded DNAs of the complete ITS regions in each genomic DNA were PCR (Polymerase chain reaction)-amplified using ITS5 (direct primer) and ITS4 (reverse primer) primers modified from Downie and Katz-Downie (1996). Each amplified DNA fragment was electrophoresed in a 1.5 % agarose gel, visualized with ethidium bromid and then excised under low wave length UV light.

**Sequencing of ITS region:** Sequencing was done using dideoxy chain termination method (Sanger *et al*., 1977) employing Sequenase (Version 2.0; USB corp.) with $\alpha$-$^{35}$S-dATP as the labeling agent. ITS4 and ITS5 were used as sequencing primers. DNA was denatureted 3 min at 100°C containing 7 $\mu$L template DNA-2 unit $\mu L^{-1}$ shrimp alkaline phosphatase 10 unit $\mu L^{-1}$ exonuclease and 100 pmol $\mu L^{-1}$ primer. Then, annealing mixture was snap-chilledon ice for 10 min. Reaction were separated electrophoretically in 6% polyacrylamide gels. Gels were dried on to whatman 3mm paper in a vacuum dryer and then exposed to X-ray film (Kodak XAR) for 2 to 4 days at room temperature.

## RESULTS AND DISCUSSION

Recent advances in DNA amplification and sequencing make the ITS regions attractive candidates for genetic markers in phylogenetic and evolutionary studies (Katz-Downie *et al*., 1999). We identified that structural and functional features of ITS conserved among *Ferulago* species through comparative sequence analysis. The entire ITS region appears to be under 700 bp in flowering plants (Fig. 1).

A direct sequencing approach can sometimes aid in detection of intragenomic repeat-type variants, which can be tentatively diagnosed by two or more nucleotide states at a site (Yeh *et al*., 1990). The length of ITS1 and ITS2 was among 439-433 ITS1 region was smaller in length than

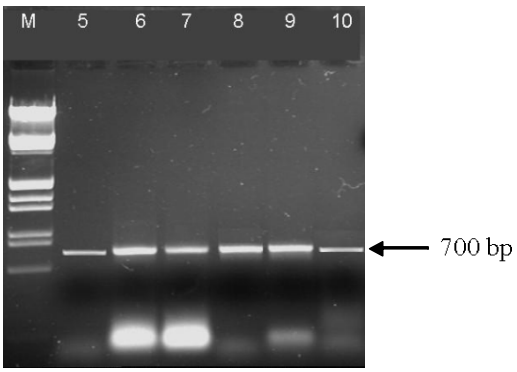


Fig. 1: The amplification product of ITS1-5.8S-ITS2 region from 5). F. asparagifolia 6). *F.* mughlae 7). *F. thirkeana* 8). *F. trachycarpa* 9). *F. silaifolia* 10). *F. confusa* M. Marker digested with *EcoRI* and *Hind III*

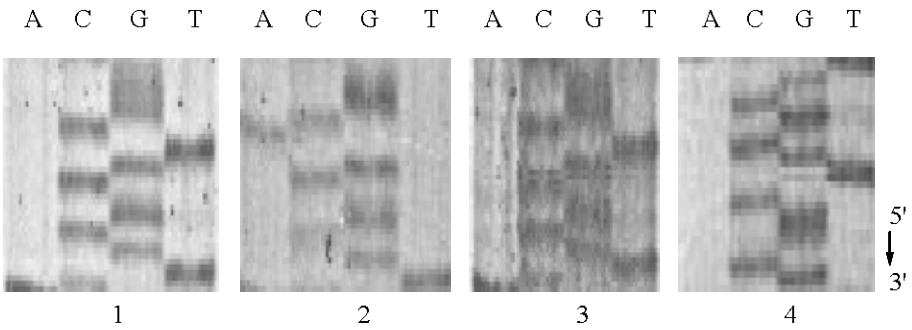


Fig. 2: GCG repeat motif in ITS1 region of *F. silaifolia* (1), *F. galbanifera* (2), *F. asparagifolia* (3), *F. thirkeana* (4)

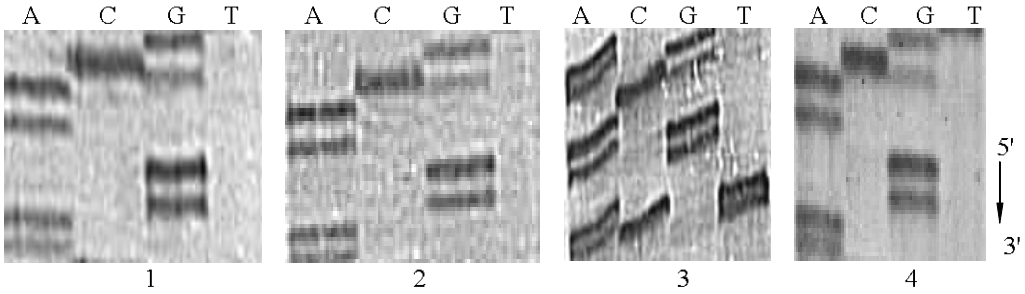


Fig. 3: 5'- GCGAAGGAA -3' conserved sequences in ITS1 of *F. asparagifolia* (1), *F. albanifera* (2), *F. silaifolia* (3) and *F. trachycarpa* (4)

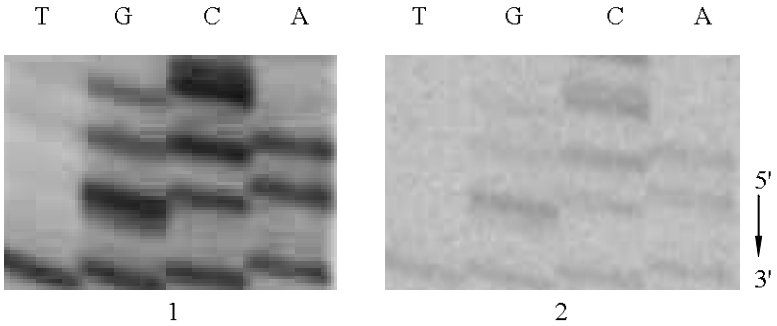


Fig. 4: 5'-GAC GAC-3' The conserved repeat motif in ITS2 of *F. asparagifolia* (1) and *F. trachycarpa* (2)

ITS2 on average. ITS2 consistently longer than ITS1 in all available sequences of *Ferulago*. ITS2 included more potentially informative characters than ITS1 (Table 2).

Both internal transcribed spacers are very G+C rich. and observed as 57.5% range for *Ferulago* (Fig. 2). Spacer segments with G+C richness may form secondary structures. G+C content may also indicate a bias in substution probabilities. GC richness is supposed to ensure thermal stability at DNA, RNA and protein levels and to be an adaptation to environment (Baldwin *et al*., 1995). Higher G+C content presumably give rise to a more stable secondary structure (Nazar *et al*., 1987).

Structural and functional features of ITS conserved among *Ferulago* species was determined through comparative sequence analysis. The small size of the ITS region (≤700 in angiosperm) and the presence of highly conserved sequences flanking each of the two spacers make this region easy to amplify, even from herbarium material (Fig. 1). The highly conserved sequence motif, GGCRY- (4 to 7n)- GYGYCAAGGAA was located in ITS1

Table 2: Sequence characteristics of the two internal transcribed spacer regions, in 10 species of *Ferulago* genus. (Qi-Square = 4.73, p = %99)

| Sequence characteristics of ITS region | ITS1 | ITS2 | ITS1 and ITS2 |
|---|---|---|---|
| Length range (bp) | 206-216 | 223-227 | 432-441 |
| Length mean (bp) | 212 | 224 | 437 |
| G+C content mean (%) | 56.3 | 58 | 57.5 |
| Number of constant sites | 185 | 160 | 468 |
| Number of variable sites | 27 | 51 | 78 |
| Number of informative sites | 15 | 22 | 37 |
| Base differences | 0.09-0.62 | 0.35-0.85 | 0.07-0.03 |

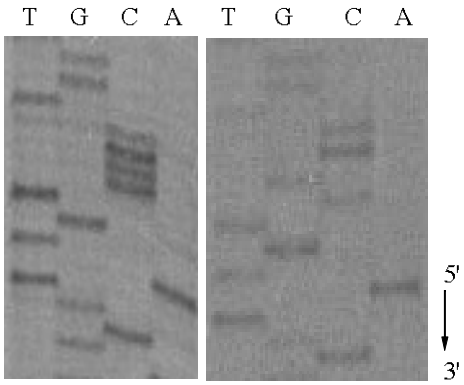


Fig. 5a: Variation among 72-84. nucleotide sites of ITS1 1) *F. sandrasica* 5'- GGTCC**C**CTGTATGCG-3', 2) *F. confusa* 5'- GGTCC**G**CTGT**A**TGCG -3'

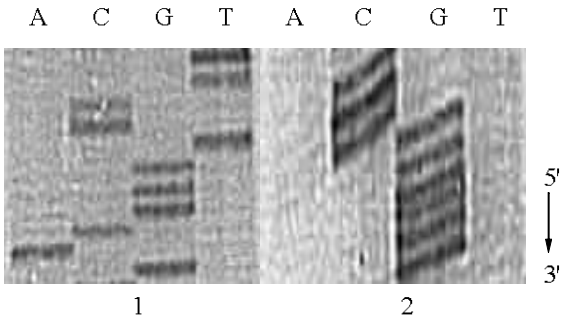


Fig. 5b: Variation among 105-114 nucleotide sites of ITS1 1) *F. galbanifera* 5'- TTCC**T**GGGCAG -3' 2) *F. silaifolia* 5'- **C**CCGGGGGG -3'

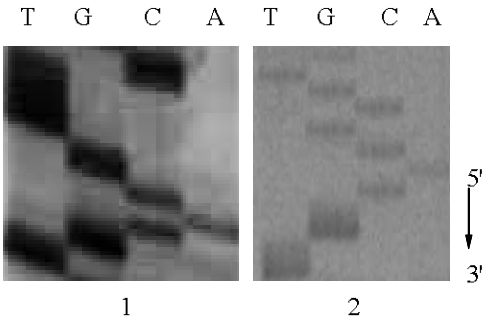


Fig. 6a: Variation among 72-85 nucleotide sites of ITS2 1) *F. asparagifolia* 5'- TTT**T**T**G**GC**A**CGGTT-3', 2) *F. thirkeana* 5'- T**GTGC**GC**A**CGGTT-3'

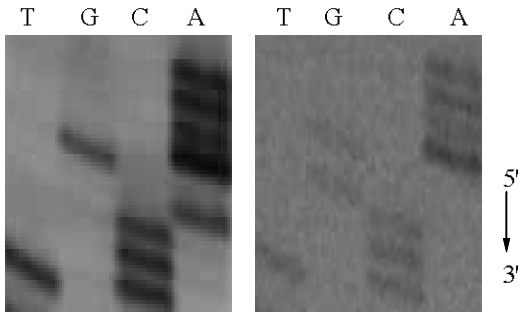


Fig. 6b: Variation among 138-147 nucleotide sites of ITS2 1) *F. galbanifera* 5'- AAAAGGCCCT-3', 2) *F. asparagifolia* 5'- AAAAG**A**CCCT -3'

and detected in published sequences from 88 species representing ten falimies and five subclasses of flowering plants (Downie and Katz-Downie, 1996) and is also seen at *Ferulago* species. The highly conserved sequence motif, GGCRY-(4 to 7 n)-GYGYCAAGGAA, located in ITS1 is seen between positions 147 and 157 at *Ferulago* species. The 5'-GCGAAGGAA-3' motif is predicted not to be part of a base-paired stem region and is thought to serve as a critical recognition element for rRNA processing (Fig. 3).

During the pre-rRNA cleavage pathway, the excision of ITS2, a eukaryote-specific insertion, remains the most elusive processing elements of ITS2 show the highest degree of sequence conservation. The 3'-ends of the 8 S and 12 S pre-rRNA intermediates of maturation are located precisely in the best preserved structural features. They lie in the 5'-GAC GAC-3' motif of ITS2. The 5'-GAC GAC-3' motif was conserved in ITS2 region among *Ferulago* species (Fig. 4). The preferential conservation of this functional site suggests its key role in ribosome biogenesis. The GAC GAC at the 3' terminus of the ITS is preceded by a stretch of pyrimidines ranging in length. These features are reminiscent of those of introns in mRNA genes, in which there is a 5' conserved sequence and a shorter 3' conserved sequence preceded by a run of pyrimidines (Bailey *et al*., 2003).

These processing sites could be functional targets of endonuclease depending on the *Ferulago* species. The strong conservation may be related to the positioning of the enzymatic complex with in the ITS conserved core through direct interaction. Since the transcripts of rRNA, ITS and mRNA introns share some similarity, analogies have been made between the mechanisms of their removal during RNA maturation.

The ITS consists of a mosaic of variable regions with intervening constant nucleotide blocks (Wenner *et al*., 2002; Antanovics and Ellstrans, 1988). ITS variants within individuals that differ by an insertion or deletion of one or more nucleotides can be more readily detected from direct sequences on sequencing gel (Fig. 5 and 6).

Mutation at ITS positions stem formation (via intrastrand nrRNA pairing) may necessitate compensatory mutations at directly opposing sites to maintain structural integrity and proper functionary of the molecule. Like

most other angiosperm ITS sequences, these regions have evolved primarily by point mutations, judging from the high levels of ITS sequence divergence between species (Baldwin *et al*., 1995). The variability of this well-known ITS region allows a differentiation between species and even sometimes between subspecies (Antanovics and Ellstrans, 1988) (Fig. 5 and 6). Resolution for phylogenetic problems amongst species is generally well correlated with the extent of variation within spacer suggested from divergence value and numbers of potentially informative bases on data ITS (Downie and Katz-Downie, 1996; Katz-Downie *et al*., 1999). Not only is the rate of nucleotide substitution appropriate for intergenic and interspecific comparisons, but the biparental inheritance of the nuclear rDNA eliminates the possibility of artifactual relationships sometimes suggested by analysis of organeller genomes.

ITS appears to provide valuable molecular markers for phylogenetic analyses of species (Soltis and Kuzoff, 1993; Doyle *et al*., 1994). Highly conserved sequences and suitable variation within ITS sequences among these *Ferulago* species raise concerns about the utility of this ITS region for phylogenetic hypothesis.

ITS sequences may not provide a valuable source of intraspecific markers for population-level studies in *Apiaceae* (Soltis and Kuzoff, 1993).

Sequence divergence values among *Ferulago* species ranged from 0.07 to 0.03 (Maras, 2005). But other groups of Apiaceae were higher or perhaps in the same range. The between two species of *Pimpinella* genus from 0.7 to 0.8% range and obtained 1.5% between species of *Lomatium* (Soltis and Kuzoff, 1993).

The presented here represent attempt to formulate more precise hypotheses about relationships of *Ferulago* species within Apioideae using evidence derived from nuclear ribosomal DNA ITS sequences. The phylogenies inferred using these molecular data reflect the species phylogeny.

ITS sequences appear best suited to comparisons of species and closely related genera and should be further explored as a promising source of nuclear phylogenetic markers within Apioideae at these levels.

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