Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2008; 33 (4) ; 163–168.

Research Article [Araştırma Makalesi]

Yayın tarihi 23 Ocak, 2009 © TurkJBiochem.com

[Published online 23 January, 2009]

An *ITS* DNA Sequence-Based Phylogenic Study of Some *Heracleum* L. (Umbelliferae) Species From Turkey's Partial Flora

[Türkiye Florasındaki Bazı *Heracleum* L. (Umbelliferae) Türlerinin ITS DNA Dizilerini Temel Alan Filogenik Bir Çalışma]

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[Received: 16 July 2008, Accepted: 23 October 2008]

Kayıt tarihi: 16 Temmuz 2008, Kabul tarihi: 23 Ekim 2008

ABSTRACT

Objective: Identification of *Heracleum* species is difficult due to minor differences of their morphologies. The objective of this study was to formulate hypotheses about relationships of *Heracleum* species within Apioideae using evidence derived from internal transcribed spacer regions of nuclear ribosomal DNA. Internal transcribed spacers may provide a valuable source of interspecific markers in Apiaceae. These regions appear well suited to comparisons among related species and/or closely related genera.

Methods: Dried seeds of seven *Heracleum* species were collected. Total genomic DNA were isolated from endosperm layer of seeds collected for each species. Double stranded DNAs of the complete ITS regions in each genomic DNA were amplified. ITS4 and ITS5 regions of nuclear ribosomal DNA were sequenced.

Results: Phylogenetic trees were obtained using ITS DNA sequence data of *Heracleum* species. Sequences were potentially phylogenetically informative. ITS2 included more potentially informative characters than ITS1.

Conclusion: In the Gene Bank, there is limited sequence data for the studied species. These data may help better understand the phylogenetic relationship of some *Heracleum* species of large and taxonomically complex Apiaceae. These results are especially important for Apioideae, which has served as an important system in many evolutionary studies

Key Words: Apiaceae, *Heracleum*, ITS sequences, molecular phylogeny, nuclear ribosomal DNA Turkish flora, Umbelliferae

ÖZET

Amaç: *Heracleum* türlerinin tanımlanmaları, morfolojilerindeki küçük farklılıklardan dolayı zordur. Bu çalışmanın amacı Apioideae içinde *Heracleum* cinsine ait türlerin akrabalık ilişkisi konusunda Nüklear ribozomal DNA'nın transcribe edilen dizilerinden (ITS) sağlanan veriler kulanılarak hipotezler üretmektir. ITS dizileri Apiacea'de çok değerli türler arası bir markırdır. Bu bölge yakın akraba cins veya türler arası karşılaştırmalara çok uygundur.

Metod: *Heracleum* cinsine ait yedi türün kuru tohumları toplandı. Her bir türün tohumlarına ait endosperm tabakasından total genomik DNA izole edildi. Genomik DNA'daki ITS bölgeleri çoğaltıldı. ITS4 and ITS5 nüklear ribozomal DNA bölgelerinin dizileri çıkartıldı.

Bulgular: ITS DNA dizi verilerini kullanarak *Heracleum* türlerinin filogenetik ağacı çıkartılmıştır. ITS dizileri filogenetik olarak bilgi vericidir. ITS2, ITS1'den daha bilgi vericidir.

Sonuçlar: Gen bankasında sınırlı sayıda nükleotid dizi verileri yeralmaktadır. Bu veriler çok geniş ve taksonomik olarak çok karmaşık olan Apiaceae familyasının ait bazı *Heracleum* cinsi türlerin tarihsel akrabalığını daha iyi anlamaya yardım edebilir. Bu sonuçlar çoğu evrimsel çalışmalarda önemli bir sistem olarak hizmet eden Apioideae için özellikle önemlidir.

Anahtar Kelimeler: Apiaceae, *Heracleum*, ITS dizileri, moleküler filogeni, nükleer ribozomal DNA, Türkiye florası, Umbelliferae

Introduction

Apiacea (Umbelliferae) is one of the best known families of flowering plants, and includes many edible plant families (e. g., carrot, parnsnips, parsley, celery, fennel, dill, anise, cumin), because of its characteristic inflorescences and fruits and the distinctive chemistry, reflected in odor, flavors an even toxicity of many of its members. This family is very large and taxonomically complex. Fruits of Apioideae exhibit extreme variation in over all forms and details and their structures have been extensively relied upon various classifications at all taxonomic levels. Characters of the fruit include its general shape, degree of compression, the presence or absence of wings, ridges, hairs or spines, the form and arrangement of spines and ridges (1).

Heracleum (Umbelliferae tribe Tordylieae) L. is the largest genus in its tribe. It consists of 90-120 species. 24 species are known from Turkey, nine of which are endemic. (2,3). In traditional medicine, some *Heracleum* species are used as antipyretic, carminative, digestive and also as a flavouring agent and spice for foods (3).

This genus is perennial or monocarpic, tall or dwarf herbs and often aromatic plants. Sepals are minute. Petals are white or sometimes pale greenish. Fruits of *Heracleum* L. genus are spiny or small glabrous ones. The fruit compressed laterally at right angels to the comissural plane, each mericarp commonly bears five primary, longitudional ribs or ridges that contain the vascular bundles: three dorsal and two marginal (or lateral), with the ribs filiform or awinged (1).

However, despite this wealth of information, morphological differences are not enough for separation of taxa on sectional level. There has been little speculation of phylogenetic relationships within the genus. Because many of its representatives are rare plants known from limited number of habitats (2). Turkey is an acknowledged centre of biodiversity in the middle-sized Umbelliferae genus *Heracleum*, and most probably, the main area of its origin and primary diversification (4). Genus is worth of further studies in Turkey. More precise hipoteses were formulated about diverse species comprising *Heracleum* genus. This goal is especially important for Apioideae, which has served as an important system in many evolutionary studies.

Nuclear ribosomal RNA genes provide markers for retrieving phylogeny at a variety of taxonomic levels (5). The nuclear ribosomal RNA genes of higher plants are organized in long, tandem repeating units (6). Each repeat unit consists of a single transcribed region for the 18 S, 5.8 S and 28 S ribosomal RNA's two small internal transcribed spacers (ITS1 and ITS2) and a large external nontranscribed intergenic spacer (IGS) (7,8).

ITS region of nuclear ribosomal DNA (nrDNA) contains an evolutionary highly conserved sequences and suitable variation within sequences. This region was appeared to be useful for phylogenetic analysis of angiosperms. ITS plays a role in ribosomal maturation and processing of small and large-subunit rRNA's (9,10,11). The evolutionary origin of the ITS is considered to be intron-like structure (12).

The entire nrDNA repeat unit is present in up to many thousands of copies arranged in tandem repeats at a chromosomal locus or at multiple loci. This high copy number promotes detection, amplification, cloning and sequencing of nrDNA. The small size of the ITS region and the presence of highly conserved sequences flanking ITS make this region easy to amplify, even from herbarium material. The gene family undergoes rapid concerted evolution via unequal cross-over and gene conversion (13,14,15).

Since many of the species of *Heracleum* are rare or endemic, further studies including species identification via molecular markers is needed (2). In this study, the phylogenic tree construction of this perennial *Heracleum* genus collected in Northeast, Northwest, and Central Turkey was attempted using their ITS sequence data.

Materials and Methods

Plant Material Collection

Seeds of 7 *Heracleum* species collected from Turkey were indicated below table 1. These plant materials were kept -20 °C.

DNA Isolation

Endosperm layers were removed from dried seeds of seven *Heracleum* species (7,16,17). Total genomic DNA were isolated from fourteen of endosperm layer from seeds collected for each species.

Amplification of ITS Region

Double stranded DNAs of the complete ITS regions in each genomic DNA were PCR (Polymerase chain reaction)-amplified using (direct primer ITS5: 5′-GGAAGGAGAAGTCGT AACAAGG-3′) and (reverse primer ITS4: 5′-TCCTTCCGCTTATTGATATGC-3′) primers modified from (17) White et al. (1985) and (7) Katz Downie and Downie (1996). Each PCR reaction cycle proceeded as follows: (i) 1 min. 94 ºC to denature the double stranded template DNA; (ii) 1 min. 53 ºC to anneal primers to single stranded template DNA and (iii) 1 min. 72 ºC to extend primers. PCR reaction achieved as 35 thermal cycles and followed 10 min. at 72 ºC extension period to complete unfinished DNA strands. Each amplified DNA fragment was electrophoresed in a 1.5 % agarose gel, visualized with ethidium bromide, then excised under low wave lenght UV light and sequenced.

Sequencing of ITS Region

Sequences were obtained using the ABI 310 DNA sequencer of Middle East Technical University Central Laboratory. ABI trace files were edited and contigs assembled using chromas (version 1.45) software programme.

Table 1. Origin of samples and Gene Bank accession numbers of ITS1 and ITS2 sequence of Heracleum species.

Species	Locality	Gene Bank Accession No.
H. pastinacifolium Koch.	Karabük-Karaağaç, Keltepe, 1800-1900 m. Rocky areas.	ITS1: AM168434 ITS2: AM167908
H. trachyloma Fisch & Meg.	Kars-Sarıkamış, 1900 m. Moist places, valley margins	ITS1: AM167909 ITS2: AM167910
H. sphondylium L.	Ankara-Kırıkkale, 900 m. Road margins, valley margins.	ITS1: AM167911 ITS2: AM167912
H. antasiaticum Manden.	Erzurum-Aşkale, 1270 m. 38 km. Soğuksu bridge.	ITS1: AM167913 ITS2: AM167914
H. sosnowskyi	Rize-Çamlıhemşin, 400 m. Water side. Moist leafes	ITS1: AM167915 ITS2: AM167916
H. persicum Desf.	Ağrı-Eleşkir, 2060 m.	ITS1: AM167917 ITS2: AM167918
H. crenatifolium Boiss.	Konya-Hadim, 800 m. Valley margins	ITS1: AM167919 ITS2: AM167920
F. asparagifolia Boiss.	İzmir, Selçuk, Efes ruins,	ITS1: AJ972387 ITS2: AJ972388

Phylogenetic Analysis

ITS data matrix was occured with MacClade 4. 03. (18). The resulting data matrix was analyzed by assuming unordered character states using PAUP 4.0b10. (19) with Machintosh performance 6320 computure. All heuristics searches were replicated 500 times with random addition sequence and tree bisection-reconnection (TBR) branch swapping. Initially all searches were performed using equal character weighting. Bootstrap analyse were performed using PAUP 4.0b10. (19) to assess the degree of support for particular branches on the strict consensus tree. Pairwise nucleotide differences of unambiguously aligned positions were determined using the Distance matrix option in PAUP. In the phylogenetic analyses, all gaps were treated as missing data. Transition/transversion ration were calculated using MacClade 4.03. (18). The amount of phylogenetic information in the parsinomy analyses was estimated using the consistency index (20), homoplasy index and retention index (21).

Results

Sequence Analysis

Accession numbers of ITS1 and ITS2 sequence of *Heracleum* species were obtained from Gene Bank. ITS1 and ITS2 sequences of these *Heracleum* species from Turkey were published for the first time at National Center for Biotechnology Information. Gene Bank accession numbers of ITS1 and ITS2 for seven species of *Heracleum* genus are provided in Table 1 and their characteristics summarized in Table 2. On average, ITS1 is 219 bp and ITS2 is 224 bp in size.

ITS1 pairwise divergence values are similar on average to those of ITS2 in *Heracleum*. ITS1 gave divergence values ranging from 0.4 to 0.10 % of nucleotides, and from 0.1-0.8 % in ITS2. Sequences were potentially informative phylogenetically. A greater proportion of ITS2 containing alignment part of the same sequence was excluded from the study during cladistic analysis. The excluded sequences do not have evolutionary variation and are the conserved sequences. ITS2 included more potentially informative characters than ITS1 (Table 2).

In direct pair wise comparisons of unambiguous positions among all *Heracleum* species, base differences ranged from 0.11 to 0.92 of nucleotides in both spacer regions. The lowest value was calculated between *H.sosnowskyi* and *H. persicum* Desf. with 0.1. The highest pairwise divergence was determined as 0.85 between *H. sphondylium* and *H. persicum*. Percent pairwise sequence divergence ratios of the combined ITS1 and ITS2 sequences from the DNA's of seven *Heracleum* species using PAUP 4.0b10 are 0.8-8 %. Transition/transversion ratio is 0.9 (Table 2).

In this study, the extent of G+C of ITS is rich, and observed as 57 % range for *Heracleum* species (Table 2). Spacer segments with G+C richness may form secondary structures. G+C content may also indicate a bias in substitution probabilities. GC richness is supposed to

Table 2. Sequence characteristics of the two internal transcribed spacer regions of species of Heracleum genus. (Qi-Square:4.73, P: %99).

Sequence characteristics of ITS region	ITS1	ITS2	(Combined ITS) ITS1 and ITS2
Length range (bp)	219	224-225	444-443
Length mean (bp)	219	224	444
G+C content mean (%)	57	50	57
Percent pairwise sequence divergence	0.3-9	1-8	0.8-8
Transition/transversion ranges	1	1	0.9
Numbers of informative sites	30	27	57

ensure thermal stability at DNA, RNA levels and to be an adaptation to environment (12,14).

Phylogenetic Analysis

Strict consensus trees from the combined ITS1 and ITS2 sequence data at Figure 1 placed *H. antasiaticum* Manden., as sister to *H. pastinacifolium* Koch., *H. tachyloma* Fisch &Meg., these combined three species were shown as a monophyletic group. *H. pastinacifolium* Koch. is the most advanced species for these three strict consensus trees. This species is perennial and not aromatic with a collar of broad flat fibres. It prefers rockier habitats than the other species. It founds on slopes with high humidity and extremelly variable in indumentum. It has a wide distribution area comprising Armenia, Azerbaijan, Southern Estern Anatolia (4,2,22).

Discussion

Like most other angiosperm ITS sequences, these regions in DNAs of *Heracleum* species have evolved primarily by point mutations, judging from the high levels of ITS sequence divergence between species (14). In all flowering plants, ITS1 and ITS2 are each less than 300 bp (23). On average, ITS2 is longer than ITS1 by ~17 bp (Table 2) according to ITS matrix sequenced.

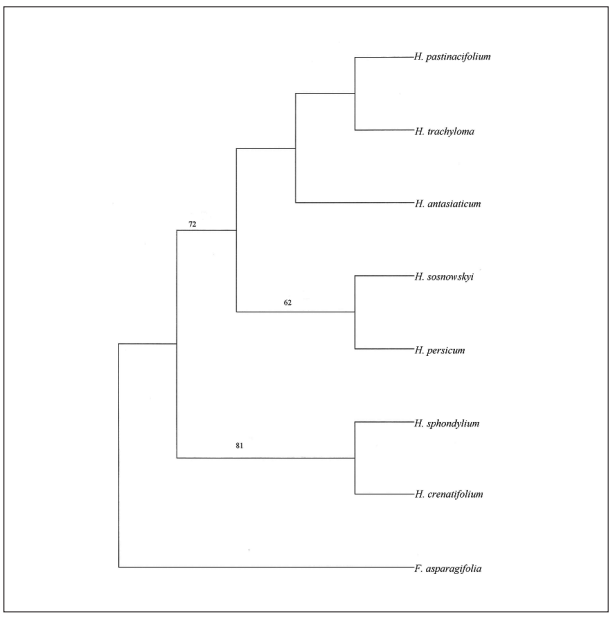

Figure 1. Strict consensus of parsimonious trees derived from equally weighted parsimony analysis of combined ITS1 and ITS2 sequences. Numbers above the branches indicate bootstrap values.

The highly conserved sequence motif, GGCRY- (4 to 7 n)- GYGYCAAGGAA, located in ITS1 and detected in published sequences from 88 species representing ten families and five subclasses of flowering plants (7), is also seen between positions 147 and 157 in ITS1 sequences for *Ferulago* Koch. 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 recognation element for rRNA processing (24).

G+C content in ITS1 is roughly similar to ones in ITS2. This similarity probably reflects some degree of co-evolution of ITS1 and ITS2 sequences as suggested by evidence that both spacers are involved in maturation of large subunit rRNA. Mutations at ITS positions involved in stem formation (via intrastrand nrRNA pairing) may necessitate compensatory mutations at directly opposing sites to maintain structural integrity and proper functioning of the molecule (25,11). 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 ITS data (7,15).

ITS appears to provide valuable molecular markers for phylogenetic analyses of species (5,26). Highly conserved sequences and suitable variations within ITS sequences among these *Heracleum* species raise corcerns about the utility of this ITS region for phylogenetic hypothesis. The greatest part of sequence variation can be explained as a result of combination of two evolutionary events: inversion and duplication (2). In this study, complementarity of ITS1 and ITS2 data sets was indicated by more complete and robust resolution. (2) Logacheva et al. explored phylogenetic relationships of West Asian *Heracleum* species using data from sequences of ITS1 and ITS2 region. They have proven to be parsimony-informative of these regions of nuclear ribosomal DNA. ITS sequences may not provide a valuable source of interspecific markers for population-level studies in *Apiaceae* (5). These regions appear well suited to compari-

sons among related species and/or closely related genera. From these data, one might conclude that the common ancestor of the *H. pastinacifolium-H. trachyloma* and *H. antasiaticum* diverged from one ancestral type. *H. sphondylium-H. crenatifolium* and *H. sosnowskyi-H. persicum* Desf. have the short branch lengths between nods relative to the lengths of terminal branches. *H. sosnowskyi-H. persicum* are evolutionarily distant from all other species of the genus. This could merely be because of rapid evolution producing a long branch; it might possible reflect an ancient isolation of the lineage. *F. asparagifolia* was selected as outgroup for this analysis. *H. pastinacifolium* was the most advanced species of all *Heracleum* genus (Figure 1.). (2) Logacheva et al. reported that West Asian *Heracleum* was a polyphyletic genus, as its species fall in to two different clades.

Sequence divergence values among *Heracleum* species ranged from 0.8 to 8 %. But other groups of Apiaceae were higher or perhaps in the same range. The pairwise divergence between two species of *Pimpinella* genus from 0.7 % to 0.8 % range, occured from 3 to 7 % between *Ferulago* species (24) and obtained 1.5 % between species of *Lomatium* (5). Relationships derived from parsimony analysis of cpDNA, rpo C1 intron sequences are considerably less resolved than, relationships derived from ITS sequences for phylogeny of Apioideae (7). Logacheva et al. used the sequences of chloroplast psbA-trnH intergenic spacer. This region has been found very variable at interspecific level (2).

Of special ecological and economical interest are those species, which have been introduced by humans and rapidly spread in their new environment. Some invasive species threaten biodiversity in natural habitats by displacement of native biota through competition, hybridization or predation and effects of invasive species are not only ecological but also economical. The northern hemispherical genus *Heracleum* has its center of diversity in the mountain regions of Asia (SW, Middle, The Caucasus), Europe (W, Central, SW, S, SE, E), and Africa (N, NW) (1). *H. pastinacifolium* is distributed southern and southern east Turkey and has wide distribution areas comprising western, east and middle Anatolia, Balkans, and Armenia. *H. crenatifolium* rests in North Anatolia, being narrow endemics. However, this case results from the widespread habitat preference and biogeographic locations of *H. sosnowskyi*. *H. pastinacifolium*, the most advanced species of all *Heracleum* genus, was collected from North west Anatolia at 2000 m height. This species is variable complex and allopathic and has subspecies from North-east Anatolia up to 3500 m height levels.

As a result, *Heracleum* species share distribution areas approximately at altitudes of 1000 - 2000 m. Habitat preferences of these species differ from moist valley margins to high mountinous areas. Their biogeographic locations change from East and North-East to North and North-West Anatolia. *Ferulago* W. Koch as a out group is a medium sized genus of Umbelliferae comprising about 45 species distributed across part of Europe (W, Central, SW, S, SE,) Asia (SW, Middle, The caucasus) and Africa (N, NW) (27).

The data presented here represent an attempt to formulate more precise hypotheses about relationships of *Heracleum* species within Apioideae using evidence derived from nuclear ribosomal DNA ITS sequences. The phylogenies infered using these molecular data and their shared life history 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.

These results will provide a resolution that may help a through understanding of the historical relationships within this large and taxonomically complex family of Apiaceae.

Acknowledgements

I acknowledge Professor Doctor Ergin Hamzaoğlu at University of Bozok for collecting sample species. I thank Middle East Technical University Central Laboratory in Ankara for help automated DNA sequencing.

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