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# Genetic Diversity and Phenetic Relationship of Dill (*Anethum graveolens* L.) by *rps*16-*trn*K DNA Sequences

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Dill (*Anethum graveolens* L.) is an annual herb with a long history and it is mainly used as a spice and as a medicine that is effective as a digestive aid, a sedative, and a narcotic, and that helps remove bad breath. Dill grows wild in the districts along the shores of the Mediterranean Sea, West Asia, China, and Korea. An estimate of the phylogenetic relationships within dill accessions in 20 countries was inferred using data from the *rps*16-*trn*K3-intergenic spacer. The aligned data sets for dill ranged from 747 to 779 nucleotides (bp) as a result of the differences in the insert/delete nucleotides. The sequence variation within the dill accessions was mostly due to nucleotide substitutions, although several small insertions and deletions can be found. Among 100 accessions from 20 countries, the Eastern Asia accessions were more closely related to the North American accessions than to the Central Asia and European accessions. Although some accessions were not congruent completely with geographical locations, the dill accessions with *rps*16-*trn*K analysis resulted in plants with better-resolved clades.

**Key words** : Dill (*Anethum graveolens* L.), *rps*16-*trn*K, phylogenic tree, sequence variation, accession

## Introduction

Dill (*Anethum graveolens* L.) is one of widespread vegetable herbs belonging to the family Apiaceae (Umbellifera). Dill is also called Hongwhoa or Syrah. Wild and weedy types of dill are widespread in the Mediterranean basin and in West Asia. It is suggested that dill originated from central Asia [18]. Similarly, Grieve [5] suggested that dill originated within an area around the Mediterranean and the South of Russia. Dill has been cultivated for thousands of years as a spice and medicinal purposes. For example, dill was found in mounds of ancient Egyptian history and in the Neolithic period in Switzerland. Dill has spread expanding the territory of the Roman Empire to many countries in the end of the first century BC.

This species contains biologically active constituents including carvone, limouene, dillapiole, borgaptene, umbelleprenin, and ɣ-sitosterole. In the central Asian countries, dill has been used as spices, stimulant, and carminative. Young leaves of dill can be edible. Essential oil extracted from the seeds has been used as pest control, pickles, and preservatives [4].

Recently, molecular methods have been used for the identification and evaluation of genetic diversity within dill accessions at the plant population levels [14, 15]. These results may provide clues to the spread process of dill.

Molecular markers which reveal extensive polymorphism are suitable for discriminating closely related genotypes [11]. However, RAPD (random amplified polymorphic DNA) has a problem of limited repeatability, with the confounding factor that repeating DNA sequences are often amplified [9].

The *rps*16-*trn*K region in chloroplast DNA usually shows sequence conservation in the regions flanking both *trn*L exons, whereas the central part is highly variable [13]. Within the intergenic spacer, no secondary-structural elements have been found, which could serve as splicing points, including that *rps*16-*trn*K are probably co-transcribed [3, 8]. A general feature of cpDNA spacer regions is the occurrence of indels that can be derived from either deletion or duplication of adjacent sequences or occur in non-repetitive regions of the spacer [6].

We analyzed intraspecific phylogenic relationships within the worldwide accessions in *A. graveolens* and to compare our results with those of previous studies of this species.

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## Materials and Methods

### DNA extraction, gene amplification and sequencing

All one hundred samples (five accessions per nation) of dill accessions were obtained from National Agrobiodiversity Center, National Academy of Agricultural Science (Suwon, Gyonggi Province in Korea) and the center collected the samples from 20 countries. Genomic DNA was extracted from fresh leaves after germinating using the DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol. Total DNA was precipitated with ethanol (-20℃), centrifuged for 30 minutes, washed in 70% ethanol to remove excess salts. DNA pellet was dried and then re-dissolved in 100ul TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). DNA was checked for shearing and concentration by agarose electrophoresis and fluometry, respectively.

Specific primers (*rps*16F:5′-AAAGGKGCTCARCCTACARGAA-3′, *trn*K5′R: 5′-TACTCTACCRTTGAGTTAGCAAC-3′) were used to amplify the entire length of the sequences for the *rps*16-*trn*K gene by polymerase chain reaction (PCR) using standard techniques at a 2.5 mmol/l $MgCl_2$ concentration.

PCR materials (50 ul volume) included 50 ng of genomic DNA, 100 uM of each dNTP, 0.2 uM of each primer, 1x enzyme buffer, and 2 unit of Taq polymerase. The amplification profile was 28 cycles of 94℃ for 30 sec, 42℃ for 60 sec, 72℃ for 60 sec, preceded by an initial denaturation at 94℃ for 90 sec and followed by a final extension at 72℃ for 5 min.

PCR products were separated on 2.0% agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). The amplified fragments were cloned into a bluescript vector and sequenced using ABI Prism 377 Sequencer (Applied Biosystem, USA). At least five clones of each accession were analyzed.

### Alignment and phylogenetic analysis

The chromatogram output for each sample was edited using the software Sequence Navigator 1.0.1 (Applied Biosystems Inc.), and the sequences were manually aligned. New sequences obtained in this study were deposited in GenBank.

An alignment was calculated using the MULTIPLE ALIGNMENT MODE of the Crustal X program. Phylogenetic relationship was estimated by MEGA5 version with maximum parsimony (MP) algorithm [17]. The MP was inferred using heuristic search, branch-swapping options and tree bisection-reconnection. Tajima's neutrality test [16] was estimated using MEGA5. Bayesian analysis was performed using MrBayes 3.1.2 [12] on the combined matrix. We calculated the MODELTEST 3.7 [10]. The best-fit maximum likelihood model was chosen using the Akaike information criterion (AIC) [1].

Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data.

## Results

The complete sequences of *rps*16-*trn*K regions for the 100 dill accessions in the world were amplified and sequenced with PCR and primers. The aligned data sets for dill ranged from 747 to 779 nucleotides (bp) as results of differences in insert/delete nucleotides (Table 2).

Alignments of *rps*16-*trn*K regions for the dill accessions were great similarity among the accessions and the unusual *rps*16-*trn*K insert were not shown. Sequence variation within dill was mostly due to nucleotide substitutions, although several small insertions and deletions can be found. Another source of sequence divergence was length variation due to stretches of short repeated that occurred at the sequences

Table 1. The code names of *Anethum graveolens* in 20 countries

| Scientific name | Code | Nation |
|---|---|---|
| *Anethum graveolens* L. | 1 | Armenia |
| *Anethum graveolens* L. | 2 | Botswana |
| *Anethum graveolens* L. | 3 | Bulgaria |
| *Anethum graveolens* L. | 4 | Canada |
| *Anethum graveolens* L. | 5 | China |
| *Anethum graveolens* L. | 6 | Georgia |
| *Anethum graveolens* L. | 7 | Germany |
| *Anethum graveolens* L. | 8 | Greece |
| *Anethum graveolens* L. | 9 | Iraq |
| *Anethum graveolens* L. | 10 | Kazakhstan |
| *Anethum graveolens* L. | 11 | South Korea |
| *Anethum graveolens* L. | 12 | Mexico |
| *Anethum graveolens* L. | 13 | Mongolia |
| *Anethum graveolens* L. | 14 | Russia |
| *Anethum graveolens* L. | 15 | Tajikistan |
| *Anethum graveolens* L. | 16 | Turkey |
| *Anethum graveolens* L. | 17 | Turkmenistan |
| *Anethum graveolens* L. | 18 | Ukraine |
| *Anethum graveolens* L. | 19 | United States |
| *Anethum graveolens* L. | 20 | Vietnam |

Table 2. Base frequencies across accessions of 20 countries' *Anethum graveolens* using *rps*16-*trn*K

| Country | A | C | G | T | Sites |
|---|---|---|---|---|---|
| Armenia | 0.36207 | 0.14058 | 0.19496 | 0.30239 | 754 |
| Botswana | 0.35942 | 0.14324 | 0.18302 | 0.31432 | 754 |
| Bulgaria | 0.35837 | 0.15679 | 0.18445 | 0.30040 | 759 |
| Canada | 0.36278 | 0.18072 | 0.14458 | 0.31191 | 747 |
| China | 0.30331 | 0.17219 | 0.22517 | 0.29934 | 755 |
| Georgia | 0.35984 | 0.14081 | 0.18905 | 0.31030 | 767 |
| Germany | 0.35576 | 0.13842 | 0.18370 | 0.32212 | 773 |
| Greece | 0.34469 | 0.13893 | 0.18087 | 0.33552 | 763 |
| Iraq | 0.36129 | 0.13806 | 0.18065 | 0.32000 | 775 |
| Kazakhstan | 0.34581 | 0.13290 | 0.17806 | 0.34323 | 775 |
| South Korea | 0.33722 | 0.15564 | 0.20493 | 0.30220 | 771 |
| Mexico | 0.35484 | 0.14968 | 0.19097 | 0.30452 | 775 |
| Mongolia | 0.35714 | 0.15584 | 0.18312 | 0.30390 | 770 |
| Russia | 0.33030 | 0.13004 | 0.16905 | 0.37061 | 769 |
| Tajikistan | 0.34323 | 0.20387 | 0.14581 | 0.30710 | 775 |
| Turkey | 0.34275 | 0.16945 | 0.20924 | 0.27856 | 779 |
| Turkmenistan | 0.35484 | 0.13935 | 0.18452 | 0.32129 | 775 |
| Ukraine | 0.36000 | 0.13935 | 0.18065 | 0.32000 | 775 |
| United States | 0.36057 | 0.13748 | 0.18158 | 0.32036 | 771 |
| Vietnam | 0.32464 | 0.13429 | 0.21382 | 0.32725 | 767 |
| Mean | 0.34895 | 0.14985 | 0.18542 | 0.31579 | 767.45 |

of TTTTT and AAAGA.

Alignment of the DNA sequences did not require allowing gaps. Total alignment length was 779 positions, of which 39 were parsimony-informative characters, 76 variable but parsimony-uninformative, and 318 constant characters.

G + C content for dill ranged between 31.1% (Kazakhstan) and 39.7% (China) (Table 2). The base frequencies did not showed the significant difference to the accessions. These values were similar to the mean (33.5%) for the dill alignments except China accession of the *rps*16-*trn*K region.

Substitution pattern and rates were estimated under the Kimura 2-parameter model. The estimated Transition/Transversion biases (*R*) varied from 4.17 to 14.85. Under maximum likelihood fits of 24 different nucleotide substitution models, substitution from G to A was 14.85 and the reverse was 7.79 (Table 3).

BIC score was the lowest at the Kimura parameter with 14938.4 (Table 4). AICc value was the lowest at the Tamura 3-parameter with 14637.4. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well.

Number of segregating sites was 548 and nucleotide diversity (π) was 0.250. Under the neutral mutation hypothesis, the probability that the Tajima test statistic (*D*) is positive

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution

| | A | T | C | G |
|---|---|---|---|---|
| A | - | 8.83 | 4.17 | **7.79** |
| T | 9.78 | - | **6.91** | 5.13 |
| C | 9.78 | **14.64** | - | 5.13 |
| G | **14.85** | 8.83 | 4.17 | - |

Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. The nucleotide frequencies are 35.04% (A), 31.65% (T/U), 18.39% (C), and 14.93% (G). There were a total of 689 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

(0.476) is less than 0.5 (Table 5). Therefore, there may be a site at which deletion/insertion, which increases the genetic variation, is operating.

The main phylogenetic analysis revealed many distinct clades (Fig. 1). The first clade includes three accessions (China, Korea, and Vietnam). Internal nodes were not strongly supported (only 28%). The second clade included Canada and United States and sistered to Mexico. The group was sistered to Mongol and next was Georgia, Kazakhstan,

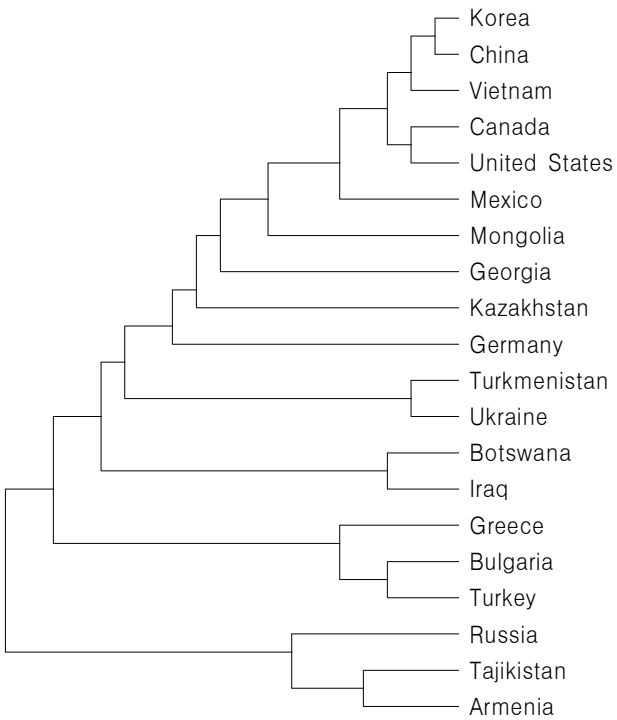


Fig. 1. The maximum parsimonious tree for 20 countries' *Anethum graveolens* based on *rps*16-*trn*K analysis using MEGA5.

Table 4. Maximum likelihood fits of 24 different nucleotide substitution models

| Model | Parameters | BIC | AICc | *lnL* | (+*I*) | (+*G*) | *R* |
|---|---|---|---|---|---|---|---|
| T92+G | 40 | 14938.368 | 14637.368 | -7278.564 | n/a | 0.64 | 0.86 |
| T92+G+I | 41 | 14947.899 | 14639.380 | -7278.565 | 0.00 | 0.64 | 0.86 |
| HKY+G | 42 | 14962.878 | 14646.840 | -7281.288 | n/a | 0.64 | 0.85 |
| TN93+G | 43 | 14970.828 | 14647.271 | -7280.498 | n/a | 0.64 | 0.85 |
| HKY+G+I | 43 | 14972.406 | 14648.850 | -7281.287 | 0.00 | 0.64 | 0.85 |
| TN93+G+I | 44 | 14980.469 | 14649.394 | -7280.553 | 0.00 | 0.64 | 0.85 |
| GTR+G | 46 | 14993.821 | 14647.711 | -7277.698 | n/a | 0.65 | 0.85 |
| GTR+G+I | 47 | 15003.355 | 14649.728 | -7277.700 | 0.00 | 0.65 | 0.85 |
| K2+G | 39 | 15231.208 | 14937.727 | -7429.750 | n/a | 0.75 | 0.75 |
| K2+G+I | 40 | 15240.732 | 14939.732 | -7429.747 | 0.00 | 0.75 | 0.75 |
| JC+G | 38 | 15255.877 | 14969.915 | -7446.850 | n/a | 0.76 | 0.50 |
| JC+G+I | 39 | 15265.415 | 14971.934 | -7446.853 | 0.00 | 0.76 | 0.50 |
| T92+I | 40 | 15275.570 | 14974.570 | -7447.166 | 0.19 | n/a | 0.73 |
| HKY+I | 42 | 15304.062 | 14988.024 | -7451.881 | 0.19 | n/a | 0.72 |
| TN93+I | 43 | 15312.998 | 14989.442 | -7451.583 | 0.19 | n/a | 0.72 |
| GTR+I | 46 | 15328.184 | 14982.074 | -7444.880 | 0.19 | n/a | 0.73 |
| K2+I | 39 | 15501.783 | 15208.302 | -7565.037 | 0.19 | n/a | 0.69 |
| JC+I | 38 | 15517.246 | 15231.285 | -7577.534 | 0.19 | n/a | 0.50 |
| T92 | 39 | 15591.777 | 15298.297 | -7610.035 | n/a | n/a | 0.70 |
| TN93 | 42 | 15625.318 | 15309.281 | -7612.509 | n/a | n/a | 0.69 |
| HKY | 41 | 15628.103 | 15319.584 | -7618.667 | n/a | n/a | 0.69 |
| GTR | 45 | 15636.553 | 15297.961 | -7603.830 | n/a | n/a | 0.70 |
| K2 | 38 | 15804.424 | 15518.463 | -7721.123 | n/a | n/a | 0.67 |
| JC | 37 | 15821.385 | 15542.943 | -7734.369 | n/a | n/a | 0.50 |

Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. For each model, AICc value (Akaike Information Criterion, corrected), Maximum Likelihood value (*lnL*), and the number of parameters (including branch lengths) are also presented. Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*). Whenever applicable, estimates of gamma shape parameter and/or the estimated fraction of invariant sites are shown. Assumed or estimated values of transition/transversion bias (*R*) are shown for each model, as well. They are followed by nucleotide frequencies (*f*) and rates of base substitutions (*r*) for each nucleotide pair. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 1 for each model. For estimating ML values, a tree topology was automatically computed.

and Germany. Clade of Turkmenistan and Ukrainewas sistered to former group. Internal node of Bulgaria and Turkey was strongly supported (71%) and sistered to Greece. Tajikistan and Armenia were formed one clade and sistered to Russia. In addition, the positions of phylogeny based on *rps*16-*trn*K analysis were not congruenced with the geographical positions.

Table 5. Results from Tajima's neutrality test for *rps*16 and *trn*K sequences of 20 countries' dills

| *m* | *S* | $p_s$ | $\Theta$ | $\pi$ | *D* |
|---|---|---|---|---|---|
| 20 | 548 | 0.795356 | 0.224187 | 0.249729 | 0.476373 |

*m*=number of sequences, *S*=Number of segregating sites, $p_s = S/m$, $\Theta = p_s/a_1$, $\pi$=nucleotide diversity, and *D* is the Tajima test statistic.

## Discussion

*rps*16-*trn*K for the one hundred accessions of dill had a total aligned length of 779 bp. Many accessions of dill contained the identical sequences over *rps*16-*trn*K gene, resulting in a single, undifferentiated clade for these samples in the phylogenetic analysis, even though many of these sample accessions had been obtained from very different geographical origins (Table 1). However, the sequences of many dill accessions differed from each other, resulting in clear delineation of all accessions in our analysis. In particular, there are many single nucleotide polymorphisms in these sequences, allowing us to distinguish accessions from other accessions based on the sequence data.

Study of genetic diversity and phylogenetic analysis in

the 135 dill accessions using molecular markers (RAPD) were reported by Suresh et al. [15]. However, their results by RAPD showed little association with the geographic origin of the collecting countries. Some accessions of same country were located in different clades of phylogenetic tree. It might be accounted for a few bands of RAPD markers (142 bands for 135 accessions). In addition, the phenetic results of RAPD were grouped into two major clusters without geographic locations. The *rps*16-*trn*K analysis resulted in trees with better-resolved clades although the present results are not congruent completely with geographical locations. Solouki et al. [14] also reported genetic diversity and morphological traits in 37 accessions of dill in Iran using AFLP (amplified fragment length polymorphism) markers. They concluded that morphological traits showed a high degree of variation among the dill accessions and molecular markers showed a low variation.

Low numbers of bands (fragments) used for RAPD or AFLP markers did not present a good relationships among accessions of dill. In addition, we are unaware of any unique anatomical or morphological traits that would support the union of these accessions of dill. As a pointed by Solouki et al. [14], dill has shown the phenetic plasticity and morphological traits may be controlled by few genes. RAPD fragments have not made the expected bands related to the morphological similarity. These similar results have been reported other species [2, 7].

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## 초록 : *rps*16-*trn*K DNA 서열에 의한 딜(*Anethum graveolens* L.)의 유전적 다양성과 유전 관계

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딜(*Anethum graveolens* L.)은 세계적으로 중요한 초본으로 양념과 약용뿐만 아니라 소화제, 진정제, 마취제, 활력제로 오래 전부터 사용되어왔다. 딜은 지중해, 서아시아, 중국과 한국 등에서 분포한다. 20개국 100계통 간 *rps*16-*trn*K3 서열을 이용하여 유전적 다양성과 유연관계를 조사하였다. 배당된 서열은 747에서 779 염기쌍으로 삽입과 결실이 있었다. 비록 일부 삽입과 결실이 발견되었지만 서열 변이는 염기 치환에 기인하였다. 동아시아 계통이 중앙아시아와 유럽보다 북미에 근연하였다. 딜의 일부 계통은 지리적 분포와 계통도에서 위치가 일치하지 않았지만 *rps*16-*trn*K로 잘 분리되었다.