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ARTICLE

# Molecular phylogenetic identification of *Artemisia* l. Species from Mongolia

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**Abstract:** The Artemisia L. genus, one of the largest genera in the Asteraceae family, consists of many medicinally important and phylogenetically unresolved species. To define the phylogenetic relationship of Artemisia species, nucleotide sequences of the nuclear *ITS* (Intergenic spacer DNA) region, chloroplast *trnL-trnF* intergenic spacer, partial sequences of plastid *rbcL* gene were identified from medicinally important 12 species included in 3 subgenera. The phylogenetic tree was constructed through the Neighbor-Joining and Maximum Parsimony analysis, respectively. The results of study revealed that the combination of the nucleotide sequences from the *ITS* and *rbcL* region was more efficient in determining the phylogenic relationship of species.

**Keywords:** *Artemisia L., Phylogenetic, Molecular marker, nuclear ITS region, chloroplast trnL-F intergenic spacer;*

## INTRODUCTION

The largest genus Artemisia of the Asteraceae family, consists of approximately 500 taxons and contains many medicinally and economically important species, such *as Artemisia annua* L. which synthesizes secondary metabolite artemisinin used for malaria treatment [2, 16]. We have a total of 104 species belonging to this genus distributed in the phytogeographical region of Mongolia [24].

Taxonomy of genus *Artemisia L.*, speciation and diversification, mainly predominant in its origin place Central Asia, remains undecided or questioned worldwide due to their high diversification, interspecific hybridization, and chromosome number or ploidy level evolutions. Artemisia L. genus, which was divided into three main subgenera earlier, was rearranged into 5-6 based on only the capitula type and florets fertility [22].

In Mongolia, the conventional classification system based on ecology, morphology, and phytogeography is used and wormwood species are classified into three main subgenera Artemisia, Seriphidium, and Dracunculus.

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Since many of the species from the Artemisia genus occupy the widest range of ecological conditions, species distributed in same area are morphologically undifferentiated with each other, or even the same species could show altered morphology in different environmental conditions, making it difficult to distinguish them through the above approaches. As performed in achieved results of phylogenetic analysis of Artemisia L. genus, subgenera Dracunculus (Besser.), Seriphidium (Besser) Poljakov. and Tridentatae (Rudberg.) are monophyletic, while Artemisia L. and Absinthium (Mill) less are polyphyletic [23]. Classification of monophyletic subgenera through any of the taxonomy criteria supports each other, while polyphyly differs among themselves.

Therefore, we aimed to reveal the subgenera classification of some representative species of Artemisia L. in Mongolia through molecular marker analysis and compare the results with that of the conventional system. At the same time, determining undistinguishable different species or morphologically differentiated same species that could not be recognized through a conventional approach is another substantial part of our study.

In the current study, a phylogenetic tree constructed through the nucleotide sequence of *ITS* (Internal transcribed spacer), chloroplast *trnL-trnF* intergenic region, and partial sequences of plastid *rbcL* gene from 12 species included in 3 subgenera.

## MATERIALS AND METHODS

### Materials:

Leaf samples of a total of 12 species such as *A. macrocephala*, *A. rutifolia*, *A. commutata*, *A. dolosa*, *A. dracunculus*, *A. xantochroa*, *A. sericea*, *A. frigida*, *A. anua*, *A. adamsii*, *A. lacinata*, and *A.tanacetifolia*, were collected from 2 (Khentei), 4 (Mongolian Dauria), and 12 (East gobi) phytogeographical regions and were used for DNA extraction.

### Methods:

*DNA extraction, PCR amplification, and sequencing.*

Total genomic DNA was extracted from the leaves of each sample with a NucleoSpin® 8 Plant II DNA extraction kit (Machery–Nagel, Germany) following the manual.

Chloroplast *trnL-trnF*, nuclear *ITS* regions, and partial sequence of *rbcL* gene were amplified through universal primers (Table 1). PCR mix consisted of 20 ng of genomic DNA, 0.5 µM of each forward and reverse primer, 1 µM of dNTP, 2,5 µM 1× buffer, and 1.0 units Taq polymerase (Pfu, Dongsheng Biotech, China) in a 50 µL volume. PCR cycling condition was subjected to 3 min denaturation at 94$^{o}$C, 30 cycles of 30-sec denaturation at 94$^{o}$C, 30-sec annealing at 50 or 60$^{o}$C(TRN, ITS), 1 min extension at 72$^{o}$C and 2 min final extension at 72$^{o}$C.

Subsequently, PCR products were purified with the PCR purification kit (PCR and DNA fragment purification kit, DSBIO), ligated in pMD18t vector, and plasmid DNA was extracted using the MINIPREP kit (Zanaspex).

Sequencing of the plasmid DNA was performed with the Zacdye 3.1 (Zanaspex) in ABI 3730xl (Thermofisher).

*Phylogenetic analyze*. The nucleotide sequence of each species was edited with Sequencher version 5.4.6 software and subsequently assembled with BIOEDIT Sequence Alignment Editor [5] version 7.0.9.0 with Clustal W multiple alignment. Phylogenetic tree was constructed with MEGA X (Molecular Evolutionary Genetics Analysis) program under version 10.0.5 [12]. Identified nucleotide sequence for nuclear *ITS*, chloroplast *trnL-trnF*and plastid *rbcL* from each sample was compared with the reference sequences registered in NCBI

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database and nucleotide variation, insertion or deletion were estimated through the Neighbor-joining and Maximum Parsimony method with 1000 bootstrap replicates.

***Table 1. Nucleotide sequences of primers used for PCR analysis***

| Primer name | Primer sequences |
|---|---|
| ITS1 | 5'TCCGTAGGTGAACCTGCGG 3' |
| ITS4 | 5' TCCTCCGCTTATTGATATGC 3' |
| trnL | 5' CGAAATCGGTAGACGCTACG 3' |
| trnF | 5' ATTTGAACTGGTGACACGAG 3' |
| rbclF | 5' ATGTCACCACAAACAGAAAC 3' |
| rbclR | 5' TCACAAGCAGCTAGTTCAGGACTC 3' |

## RESULTS AND DISCUSSION

The amplified DNA regions were sequenced in both directions and the raw sequenced data from studied species were assembled using Sequencher version 5.4.6 software (Gene codes Co.).

A total of four types of phylogenetic trees, generated from three markers singly for newly sequenced data of 12 Artemisia species included 3 subgenera of the genus Artemisia L. from Mongolia with those retrieved from GenBank (nrDNA-ITS). The remaining tree was generated by concatenating nrDNA-ITSand cpDNA-rbcl markers. Anthemis cotula L. (trnL-trF and ITS), Chrysanthemum coronarium L. (ITS) from the same tribe Asteraceae were taken by the outgroups. The details of generated trees are given below.

- cpDNA-trn (12 new sequences + 1 Outgroup sequences)
- nrDNA-ITS (12 new sequences + 11= GenBank sequences + 1 Outgroup sequences)
- cpDNA-rbcl (10 new sequences + 1 Outgroup sequences)
- nrDNA-ITS + cpDNA-rbcl (10 new sequences + 1 Outgroup sequences)

The sequence length used for phylogenetic tree construction was highly diverged 450 bp for chloroplast trnL-trnF intergenic spacer, 350 bp for nuclear ITS region, and 428 bp for rbcl gene, respectively. Local names are indicated in the brackets for species used for this study (tab.2 and fig. 3). Nucleotide sequences of ITS region from 12 species and rbcl from 10 species registered in NCBI database with corresponding accession numbers are shown in Table 2.

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***Table 2. Accession numbers for ITS and rbcL sequences of Artemisia species in NCBI***

| № | Scientific name | Local name | Accession numbers | |
|---|---|---|---|---|
| | | | *ITS* | *rbcL* |
| 1 | *Artemisia laciniata* Willd. | Salbant sharilj | MT271738 | OR687228 |
| 2 | *Artemisia annua* Linn. | Neg nast sharilj | MT271739 | OR576208 |
| 3 | *Artemisia macrocephala* Jacquem. ex Besser. | Eerem sharilj | MT271740 | OR687229 |
| 4 | *Artemisia tanacetifolia* Linn. | Maralkhai sharilj | MT271741 | OR687232 |
| 6 | *Artemisia dolosa* Krasch. | Khuviramtgai sharilj | MT271743 | OR687225 |
| 7 | *Artemisia frigida* Willd. | Khuitseg Sharilj | MT271744 | OR687227 |
| 8 | *Artemisia sericea* Weber ex Stechm. | Torgon sharilj | MT271745 | OR687231 |
| 9 | *Artemisia rutifolia* Stephan ex Spreng. | Shargal sharilj | MT271746 | OR687230 |
| 10 | *Artemisia adamsii* Besser. | Adamsiin sharilj | MT271747 | |
| 11 | *Artemisia dracunculus* Linn. | Ishgen sharilj | MT271748 | OR687226 |
| 12 | *Artemisia commutata* Bess. | Khurgan sharilj | MT271749 | |
| 13 | *Artemisia xanthochroa* Krasch. | Shar sharilj | MT271750 | OR687233 |

Many researchers have made enormous efforts in the classification of the genus with a huge number of species, based on pollen morphology [8], anatomy [1, 9], molecular differences [3, 4, 6, 7, 11, 21], cytogenetic [17, 25], and chemotaxonomy [18]. There are many economically important species that have been used for a wide range of purposes, such as food, remedies, and ornament. Out of the 104 species distributed in our country, 43 have been confirmed that could produce some useful metabolites with important medical implications [13]. In the current study, 12 medicinal \important species have been used for the identification of phylogenetic relationships through molecular marker analysis.

Publicly available trnL-trnF intergenic spacer nucleotide sequence of few Artemisia species are insufficient to compare with our newly sequenced data. Therefore, a phylogenetic tree constructed only with newly determined nucleotide sequences of that region from each species used in the study (Fig. 1). It indicates that interspecies, even in inter-genera variations at this region of DNA is relatively low. Otherwise, this molecular marker is not variable enough for making a phylogeny in the genus Artemisia L.

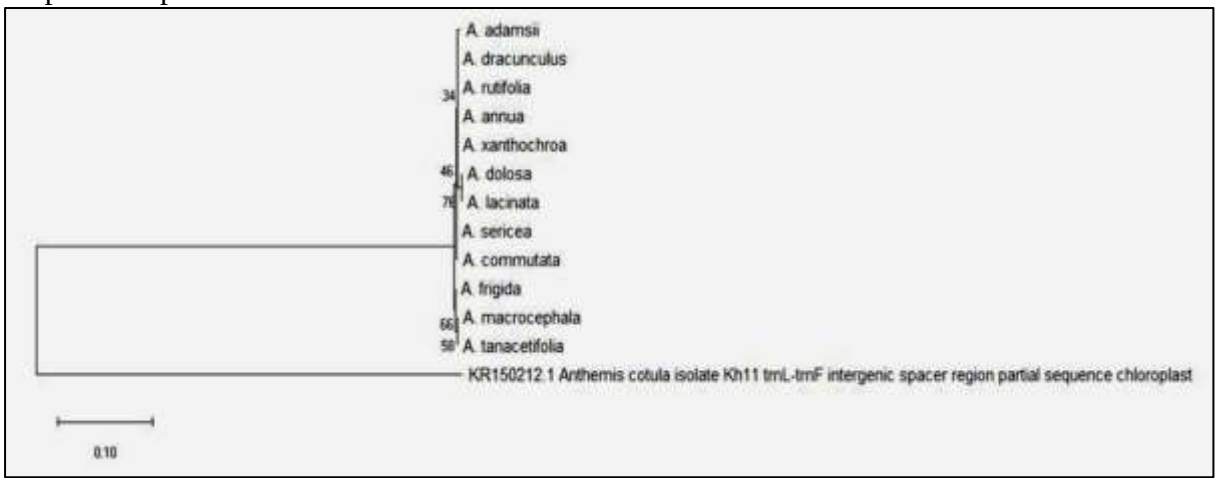


***Figure 1. Phylogenetic tree created by nucleotide sequences from chloroplast trnL-trnF region from 12 species with their matching references from GenBank. Anthemis cotula L. (Accession number KR150212.1) taken by out-group. Numbers on the nodes indicates probability of bootstrap***

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The dendrogram created by the Nieghbor-Joining method through sequences of nuclear ITS dataset revealed that there is the existence of two major groups corresponding to species with a Dracunculus, and Artemisia or Absinthim subgenera, respectively (Fig. 2). Few smaller clades were found within each of the major groups. As described by Poljakov et al [20] and Kornkven et al. [10], a genus of Artemisia was divided into 3 subgenera, such as Artemisia, Seriphidium, and Dracunculus. Later in 1974, Persson et al. [19] regrouped it and subgenera Absinthium (Mill.) was isolated from Artemisia L., resulting in four independent subgenera.

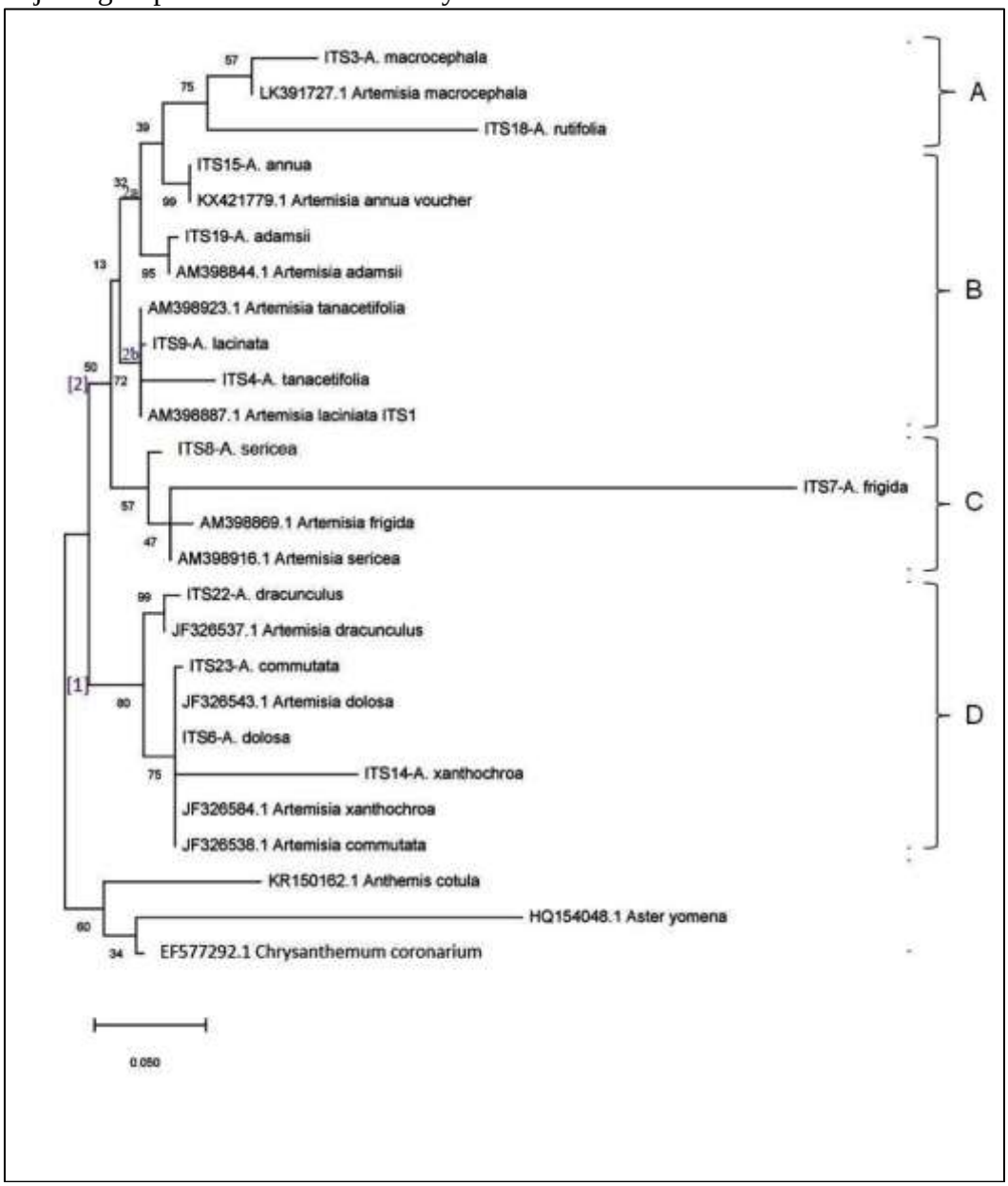


***Figure 2. Neighbor-joining phylogenetic tree based on internal transcribed spacer (ITS) sequences from 12 species with their matching references from GenBank. Anthemis cotula L. (Accession number KR150162.1), Aster yomena (Kitam.) (Accession number HQ154048.1), and Chrysanthemum coronarium L. (Accession number EF577292.1) taken by out-groups. Numbers on the nodes indicate probability of bootstrap. Local name is indicated as pronounced in the brackets for species used in this study. A and C indicates species for Absinthium, B indicates species for Artemisia,and D indicates species for Dracunculus subgenera, respectively***

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As revealed in our results, subgenera Artemisia and Absinthium were mixed together in the same major group (Fig. 2A, B), indicating that the origin of these subgenera is the same. Although species *A. annua* (Neg nast sharilj), *A.adamsii* (Adamsiin sharilj), *A. tanacetifolia* (Maralkhai sharilj), and *A. lacinata* (Salbant sharilj) from Artemisia subgenera included in the same subclade with their related reference species (accession numbers KX421779.1, AM398844.1, AM398923.1, AM398887.1), *A.macrocephala* (Eeremsharilj) and *A. rutifolia* (Shargal sharilj) from subgenera Absinthium also intervened at the same subclade (Fig. 2A,B,C). Besides, *A. Sericea* (Torgon sharilj), and *A. frigida* (Khuitseg sharilj) from Absinthium subgenera with their exemplified species (AM398869.1, AM398916.1) included separate subclade (Fig. 2C). The next major group combined *A. Dracunculus* (Ishgen sharilj), *A. commutata* (Khurgan sharilj), *A. dolosa* (Khuwiramtgai sharilj), and *A. Xantochroa* (Shar sharilj) from Dracunculus subgenera together with their corresponding data from the NCBI database (accession numbers JF326537.1, JF326538.1, JF326543.1, JF326584.1) (Fig. 2D).

Along with the above method, we also constructed the tree through Maximum Parsimony analysis with the same nucleotide sequences of identical species (Fig. 3). Generally, the structure of the constructed tree was similar to the previous one (Fig 3B and C). However, even though the species from the Absinthium subgenera united together with species from the Artemisia subgenera in the same major group, they were isolated separately in a small subclade with their matching same species (Fig. 3A). Maximum Parsimony method gave the most accurate result of the tree drawn with Tamura 3-parameter step. Thus, we used this method for our next analysis.

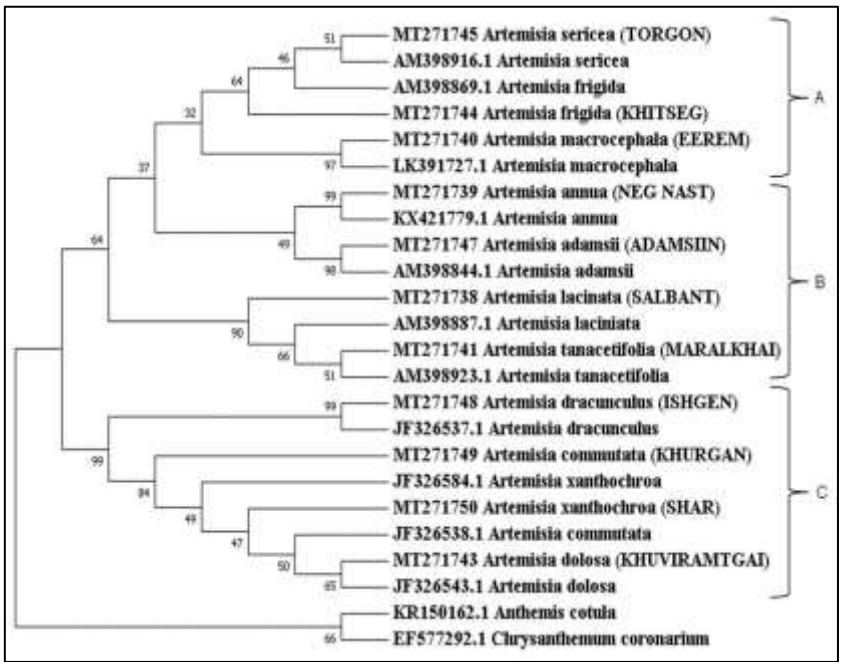


***Figure 3. Phylogenetic tree constructed through Maximum parsimony analysis based on internal transcribed spacer (ITS) sequences from 12 species with their matching references from GenBank. Anthemis cotula L. (Accession number KR150162.1), and Chrysanthemum coronarium L. (Accession number EF577292.1) taken by out-groups. Numbers on the nodes indicates probability of bootstrap. Local name is indicated as pronounced in the brackets for species used in this study. A indicates species for Absinthium, B indicates species for Artemisia, and C indicates species for Dracunculus subgenera, respectively***

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The rbcL gene is encoded by cpDNA and has been widely used for plant phylogenetic studies due to its slower rate of evolutionary change and the lowest divergence among the plastid genes in flowering plants. Accordingly, phylogeny of 10 species of Artemisia genera was confirmed by the partial sequences of rbcl gene. Species discrimination was possible with the rbcL marker and species included in Artemisia subgenus (*A. annua*, *A. lacinata*, *A tanacetifolia*) were grouped together while species from Absinthium (*A. frigida*, *A. rutifolia*, *A.macrocephala* included in same clade. However, nucleotide sequence from *A.sericeae* (species of Absinthium subgenus) were more closer to species from Dracunculus subgenus. Although Dracunculus is the most supported and resolved subgenus of Artemisia, the phylogeny tree using *rbcL* shows that *A.dolosa*, *A.dracunculus* and *A.xanthochroa* were genetically distinct. This result indicates that molecular markers or shorter DNA sequences are not sufficient enough to distinguish the phylogeny properly.

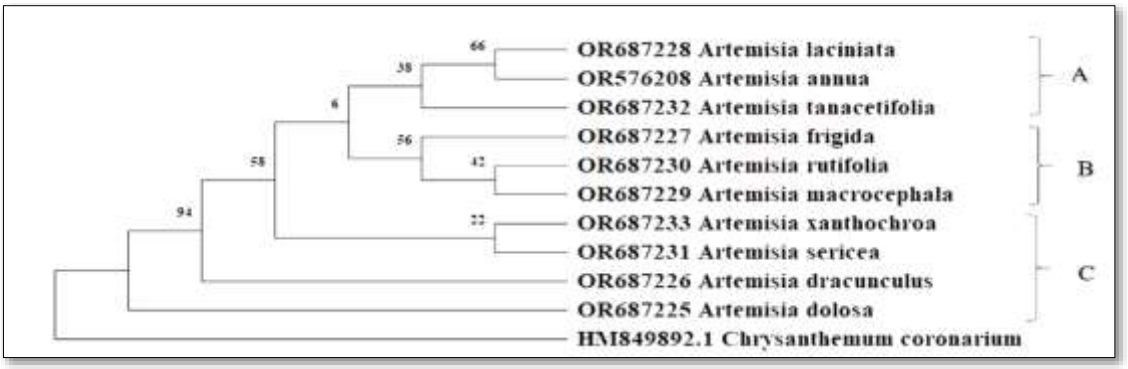


***Figure 4. Phylogenetic tree constructed through Maximum parsimony analysis based on rbcl sequences from 10 species. Chrysanthemum coronarium L. (Accession number HM849892.1) taken by out-groups. Numbers on the nodes indicates probability of bootstrap. A indicates species for Artemisia, B indicates species for Absinthium, and C indicates species for Dracunculus subgenera, respectively***

Since the discriminatory efficiency of the molecular marker ITS and *rbcL* were possible for species in the study, the combination of these markers was also used for building a phylogeny tree. The species belonging to each subgenus were undoubtedly included to the same clades (Fig. 5). Based on these results, it can be concluded that the ITS together with the *rbcL*region can be considered an effective molecular marker for describing species variation.

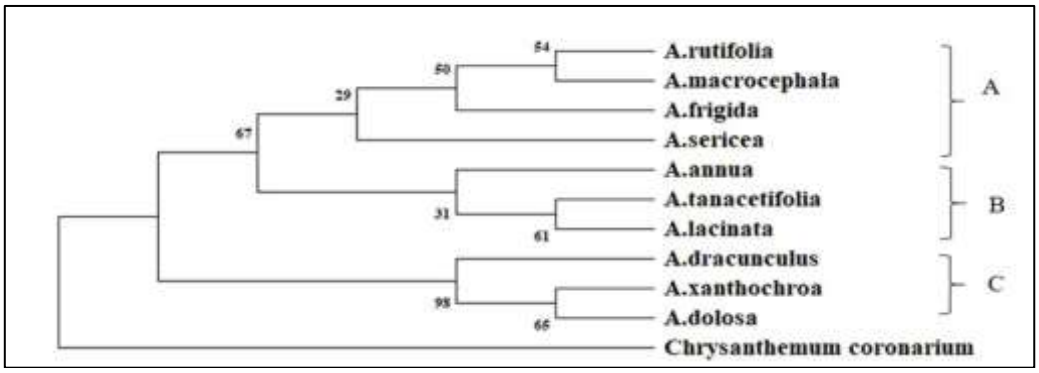


***Figure 5. Maximum Parsimony tree obtained from the co-evaluation of sequences of the ITS and rbcL regions of individuals. A indicates species for Absinthium, B indicates species for Artemisia, and C indicates species for Dracunculus subgenera, respectively***

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Morphologically, the flower receptacle of species included in the Absinthium subgenera is glabrous, while it is hairy for species from other subgenera. Capitula type and flower fertility of species from Artemisia, Absinthium, and Dracunculus subgenera are generally quire identitical with each other; Heterogamous capitula with outer florets is female and central florets are hermaphrodite and fertile, however, female florets are sterile for Dracunculus subgenera. For Seriphidium subgenera, capitula are homogamous and all florets are hermaphrodite and fertile [25]. Through the classification system, based on this morphology with the general structure of productive organs, it could be divided into 6 subgenera or sections (subg. Artemisia, Absinthium, Dracunculus, Seriphidium, Tridentatae, Pacifica) [22, 26]. However, many research results described that DNA sequence-based classification of the subgenera does not agree with that of morphology [18, 19, 24]. In our results, *A. macrocephala* and *A. rutifolia,* which are included in Absinthium subgenera by morphology-based taxonomy, were grouped in the same clade with species from Artemisia subgenera through the ITS marker sequences (Fig. 2B and 3A, B). It has also been indicated and confirmed by our results that althoughn the Absinthium subgenera is classified separately from Artemisia L. and is considered an independent subgenus through the traditional system, comparison of the DNA sequences from corresponding species does not support it. However, phylogenetic tree with combination of nucleotide sequences of *ITS* and *rbcL* clearly indicated that Artemisia and Absinthium are two distinct subgenera. Since the number of species used in this study was limited, it is important to prove this results in more species.

For subgenus Dracunculus, plenty of results from morphology or DNA differences confirmed that it is an independent subgenera[17, 18, 23, 26]. In our case, species from Dracunculus (Besser.) subgenus was also included in the same major group through the ITS marker (Fig 2C and 3C), indicating that traditional and DNA sequence-based classification issupport each other for this subgenera.

## CONCLUSIONS

Nucleotide sequences of each species used in our study were clustered together with relevant sequences registered in the GeneBank database, confirming that DNA-based molecular differences could distinguish the species difficult to identify by morphology.

The longer DNA sequences with high polymorphism is more informative for the classification of Artemisia.

## Acknowledgement

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