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The morphological, karyological and phylogenetic analyses of three *Artemisia* L. (Asteraceae) species that around the Van Lake in Turkey

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Abstract. *Artemisia* is one of the biggest genera in the family Asteraceae, with around 500-600 taxa at specific and sub-specific levels and organised in five subgenera. Due to the high number of taxa, a lot taxonomists are trying to solve the problem of its classification and phylogeny but its natural classification still has not been achieved. The aim of this study is to try to solve the problematic systematic relationship between three different *Artemisia* species growing in close proximity to each other in the light of morphological, karyological and molecular data. The roots, stems, leaves, flowers structures of the plant samples collected from different populations belong to these species were investigated within the framework of morphological studies. Additionally, the chromosome counts and karyotype analysises of these species were made and idiograms were drawn in the karyological studies. In the context of phylogenetic studies, ITS (ITS1-5.8S-ITS2) and *trn*T - *trn*L3' regions of 22 individuals belonging to 3 taxa were studied. According to results of phylogenetic anlysis, it has been found that there is completed speciation genetic isolation mechanism between the species *Artemisia spicigera*, *Artemisia taurica* and *Artemisia fragrans* that inhibit gene flow. Also *Artemisia fragrans* and *Artemisia spicigera* species are very similar to each other in terms of morphological characteristics. However, since populations of the species *Artemisia fragrans* are autopolyploid, the dimensional values of their morphological squares are larger than those of the species *Artemisia spicigera*. This study is important as it is the first molecular based study relating with some species of *Artemisia* growing naturally in Turkey.

Keywords: *Artemisia*, karyology, morphology, phylogeny, cpDNA *trn*T-*trn*L3', r-DNA ITS.

INTRODUCTION

Artemisia is one of the biggest genera in the family Asteraceae, with around 500-600 taxa at specific and sub-specific levels and organised in five subgenera (Vallès *et al.*, 2011). The majority of the members of this genus have a high economic value (Chehregani *et al.*, 2010; Hayat *et al.*, 2010). Due

to the high number of taxa, a lot taxonomists are trying to solve the problem of its classification and phylogeny but its natural classification still has not been achieved (Mcarthur *et al.,* 1981; Torrel *et al*., 1999; Torrell and Vallès 2001; Vallès *et al.,* 2003; Kurşat, 2010; Kurşat *et al.,* 2011). The genus is currently divided into five main groups [*Artemisia, Absinthium* (Mill.) Less., *Dracunculus* (Besser) Rydb., *Seriphidium* Besser and *Tridentatae* (Rydb.) McArthur] but subgeneric classification is subject to rearrangements in the light of recent molecular studies (Torrell *et al*., 1999; Vallès *et al.,* 2003).

Although a lot of investigation have been made of the genus *Artemisia,* enhancing the available morphologic and karyological data (Kawatani and Ohno 1964; Vallès 1987a; Torrell *et al*., 1999; Torrell and Vallès 2001; Torrell *et al.,* 2001; Vallès and Mcarthur 2001; Vallès *et al.,* 2001; Kreitschitz and Vallès 2003; Inceer and Hayirlioglu-Ayaz 2007; Pellicer *et al.,* 2007; Chehregani and Hajisadeghian 2009; Nazirzadeh *et al.,* 2009; Chehregani *et al.,* 2010), still the chromosome numbers of some species of the genus remain unknown or doubtful. The genus has two basic chromosome numbers, the largely predominating x=9 and the less extended x=8. x = 9 is not only the most common basic number in the genus *Artemisia*, but in the tribe Anthemideae and the family Asteraceae as well (Mcarthur and Sanderson 1999; Oliva and Vallès 1994; Schweizer and Ehrendorfer 1983; Solbrig 1977; Vallès and Siljak-Yakovlev 1997). A high percentage of *Artemisia* species are polyploid. This phenomenon is present in all of the major groups into which the genus is divided. Both basic chromosome numbers (x=8 and x=9) show polyploidy, with levels up to 12x for x=9 and 6x for x=8 (Vallès and Mcarthur 2001).

The gene regions that have been used for phylogeographic and phylogenetic inferences in plants come from the single copy portions (LSC and SSC) of the chloroplast genome, and internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA). Several molecular methods have been used to determine the genetic diversity and relationships among different *Artemisia* species, including karyotyping (Mcarthur and Pope 1979), cpDNA restriction site variation analysis (Kornkven *et al.,* 1999), polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) analysis of several genes (Lee *et al.,* 2009; Mahmood *et al.,* 2011), microsatellite (SSR) polymorphism analysis (Tripathi *et al.,* 2009; Shafie *et al.,* 2011) and Random amplified polymorphic DNA (RAPD) analysis (Mcarthur *et al.,* 1998a; Mcarthur *et al.,* 1998b; Sangwan *et al.,* 1999). Nevertheless, very few of *Artemisia* species have been verified with molecular phylogenetic studies based on nucleotide sequence data in Turkey, so far (Koloren *et al.,* 2016).

So, the aim of this study is to try to solve the problematic systematic relationship between three different *Artemisia* species growing in close proximity to each other in the light of morphological, karyological and molecular data (using of rDNA ITS and *trn*T-*trn*L3' regions sequence data). Additionally, the first molecular data for *Artemisia spicigera, Artemisia taurica*, and *Artemisia fragrans* from Turkey has been submitted to the GenBank databases.

MATERIAL AND METHODS

Morphological evaluation

Plant specimens were collected from around the Van Lake during the vegetative, flower and seed season in 2010, collected by M. Kurşat, Ş. Civelek and P.Y. Sancar. Morphological examinations consist of instant observations on the samples in the field and macroscopic and microscopic examinations on the samples that have been converted into herbarium material in the laboratory. In order to determine the minimum and maximum values of the examined characters, 10 samples were taken from each locality and measurements were made. Measurements of small structures were made with a ruler under a stereo microscope. Measurements of macroscopic structures were made using the ruler again with the naked eye. The herbarium materials of the collected samples are stored in Firat University Herbarium (FUH). The list of the examined specimens, localities, collected date and voucher numbers were given in Table 1.

Karyology

Meristematic cells of root tips are used in the caryological studies. The seeds (about 15-20 seeds for each type) were germinated on moist filter paper in petri dishes between 20-25°C. The actively growing root tips, 1 cm in length, were excised from the germinating seeds and pretreated with aqueous colchicine (0.05%) for 3-3.5 h at room temperature. Then, the root tips were fixed with Farmer (1:3 glacial acetic acid–absolute ethanol) for at least 24 h at 4°C, hydrolysed in 0.1 N HCl at room temperature for 1 min, and subsequently rinsed in tap water for 3–5 min. Then they were stained in Feulgen for 1 h and mounted in 45% acetic acid (Gedik *et al.,* 2014). Digital photographs from at least five well-spread metaphase plates from each species were taken using an Olympus BX51 microscope (Olympus Optical Co. Ltd, Tokyo, Japan), and were recorded with an Olympus Camedia C-4000 digital camera (Olympus Optical

Table 1. Information of *Artemisia* populations location in field.

Taxa	Locality	Altitude	Lat - Lon	Date	Voucher number
A. spicigera	Ant valley slope 5.5 km after passing Aktuzla	1555m	39°.21'-42°.15'	10.10.2010	Yilmaz Sancar, Kurşat and Civelek 5007,5008,5022
	Hınıs - Varto highway, 24 km before Varto roadside, slopes	1780m	39°.13'-41°.42'	10.10.2010	Yilmaz Sancar, Kurşat and Civelek 5009,5024
	Van - Hakkâri road, after 1 km of Gürpınar road separation, Çavuştepe locality roadside	1799m	38°.20'-43°.25'	25.11.2010	Yilmaz Sancar, Kurşat and Civelek 5013
A. fragrans	Kuzgun Koran Pass hills	2142m	38°.23'-42°.47'	09.10.2010	Yilmaz Sancar, Kurşat and Civelek 5001,5002,5010,5011
	Between Edremit and Gürpınar, 15 km before Gürpınar	1714m	38°.19'-43°.14'	09.10.2010	Yilmaz Sancar, Kurşat and Civelek 5003,5012
	Muradiye - Şelale location	1788m	39°.03'-43°.25'	10.10.2010	Yilmaz Sancar, Kurşat and Civelek 5005,5016
	Between Malazgirt - Aktuzla, around Nurettin village roadside slopes	1728m	38°.50'-43°.25'	26.11.2010	Yilmaz Sancar, Kurşat and Civelek 5017
	After passing Aktuzla 5.5 km, Ant Valley slopes (roadside slopes)	1555m	39°.21'-42°.15'	26.11.2010	Yilmaz Sancar, Kurşat and Civelek 5006,5019,5020,5021,5023
A. taurica	Van-hakkari highway, after 1 km of Gürpınar crossroads, Çavuştepe area, roadsides	1799m	40°.25'-43°.20'	25.11.2010	Yilmaz Sancar, Kurşat and Civelek 5004,5027,5028,

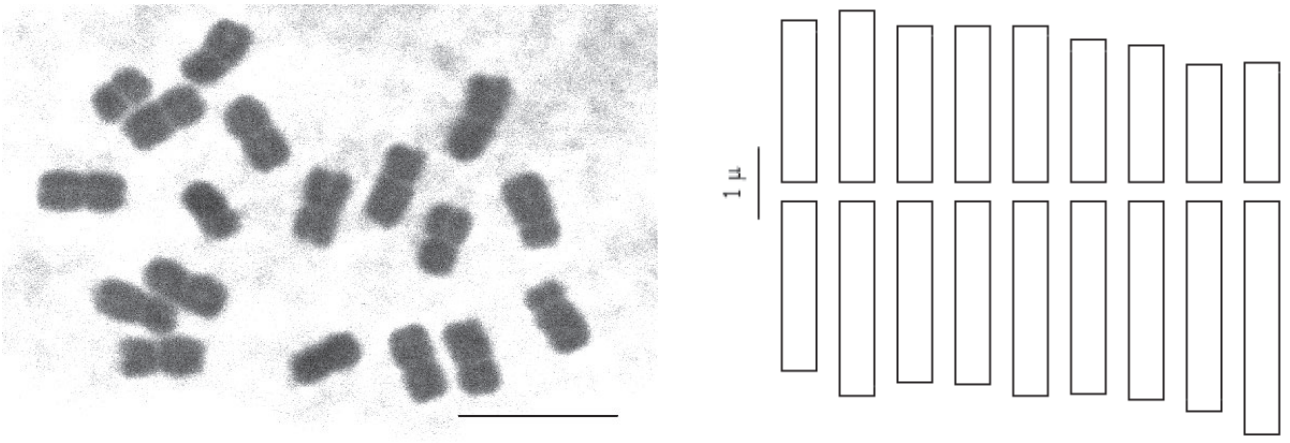

Figure 1. Somatic metaphase in *A. spicigera* (2*n*=18), and haploid idiogram (Scale bars: 1 µm).

Co. Ltd) (Figure 1). The short arm, long arm and total lengths of each chromosome were measured and the relative lengths (RL), arm ratios (AR), and centromeric indices (CI) were determined from images of selected cells. Levan *et al.* (1964) was used for the classification of chromosomes.

The number of somatic chromosomes, ploidy level, karyotype formula, morphometric parameters, A1 and A2 values (The intrachromosomal asymmetry index - A1 and the interchromosomal asymmetry index - A2) were determined for each taxa (Romero Zarco 1986) (Table 2). Idiograms of haploid chromosomes were drawn (Figure 2). The examined taxa and characteristics of somatic chromosomes are given in the results section.

Genomic DNA isolation, PCR, and Sequencing

Genomic DNA isolation was performed manually as described CTAB method by Doyle and Doyle (1987). In PCR studies conducted by using *trn*a- *trn*d primers and ITS4-ITS5 primers, *trn*T - *trn*L3' and ITS (ITS1-

Table 2. Somatic chromosome numbers (2*n*), ploidy level, karyotype formula, ranges of chromosome length, total karyotype length (TKL), and asymmetry indexes (A1, A2) of the studied taxa.

Taxon	2*n*	Ploidy level	Karyotype formula	Chromosome length range (μm)	TKL (μm)	A1	A2
A. spicigera 1	18	2*x*	1M+5m+3sm	4,94-5,56	46,72	0,27	0,04
A. spicigera 2	18	2 *x*	2M+5m+2sm	4,26-4,81	40,79	0,23	0,02
A. spicigera 3	18	2 *x*	1M+6m+2sm	4,64-5,14	43,02	0,24	0,04
A. spicigera 4	18	2 *x*	1M+6m+2sm	4,69-4,97	43,40	0,25	0,02
A. spicigera 5	18	2 *x*	2M+6m+1sm	4,55-5,50	42,28	0.26	0,03
A. spicigera 6	18	2 *x*	3M+4m+2sm	4,42-5,25	41,40	0,22	0,03
A. spicigera 7	18	2 *x*	1M+4m+4sm	4,34-5,33	44,50	0,28	0,04
A. taurica 1	36	4 *x*	3M+13m+2sm	3,48-4,07	67,31	0,22	0,05
A. taurica 2	36	4 *x*	4M+12m+2sm	4,57-5,51	92,47	0,25	0,05
A. taurica 3	36	4 *x*	4M+11m+3sm	4,44-5,50	89,70	0,23	0,06
A. fragrans 1	36	4 *x*	3M+10m+5sm	4,10-5,35	85,51	0,35	0,08
A. fragrans 2	36	4 *x*	3M+11m+4sm	3,13-3,53	59,52	0,24	0,01
A. fragrans 3	36	4 *x*	2M+14m+2sm	3,40-3,94	66,85	0,25	0,05
A. fragrans 4	36	4 *x*	2M+10m+6sm	4,81-6,29	100,08	0,26	0,07
A. fragrans 5	36	4 *x*	1M+14m+3sm	3,97-4,65	77,57	0,27	0,05
A. fragrans 6	34	4 *x*	3M+12m+2sm	3,98-4,89	75,78	0,20	0,07
A. fragrans 7	36	4 *x*	3M+13m+2sm	3,67-4,14	70,40	0,24	0,04
A. fragrans 8	36	4 *x*	1M+13m+4sm	3,43-4,75	70,15	0,29	0,08
A. fragrans 9	36	4 *x*	3M+12m+3sm	3,98-4,99	82,53	0,23	0,07
A. fragrans 10	36	4 *x*	2M+11m+5sm	3,83-4,79	76,09	0,28	0,07
A. fragrans 11	36	4 *x*	2M+10m+6sm	3,65-4,51	71,96	0,27	0,06
A. fragrans 12	36	4 *x*	3M+12m+3sm	3,40-4,49	70,52	0,27	0,08

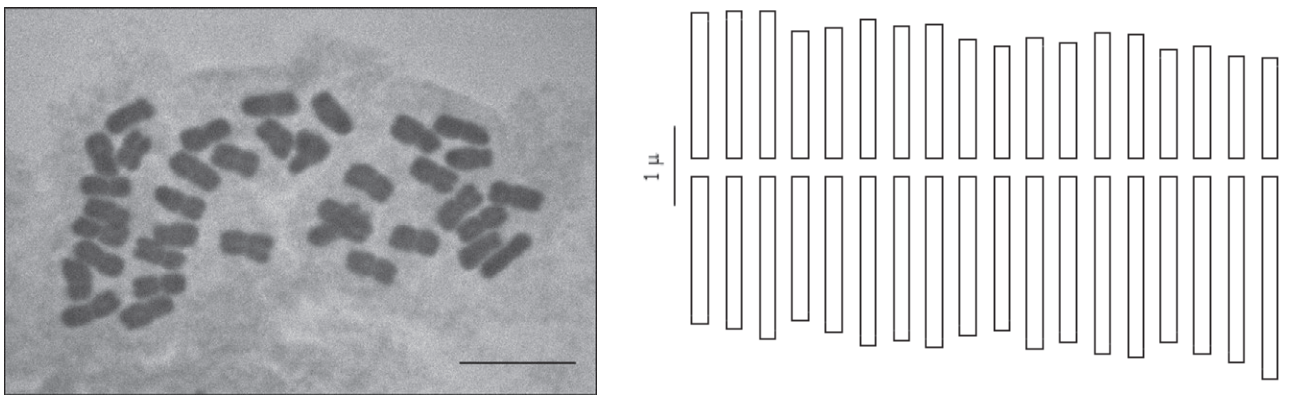

Figure 2. Somatic metaphase in *A. taurica* (2*n*=36), and haploid idiogram (Scale bars: 1 μm).

5.8S-ITS2) region for 22 samples has been multiplied (Taberlet *et al.,* 1991). The sequence of primers that were used to amplified both *trn*T - *trn*L3' region and ITS (ITS1-5.8S-ITS2) region were given in Table 3 (Taberlet *et al.,* 1991). The following protocol on a BıoRad Thermal Cycler : 2 min 95 °C initial denaturation, 35 cycles of 1 min 95 °C denaturation, 40 s 60 °C (for *trn* region) and 55 °C (for ITS region) annealing and 1 min 72 °C extension, followed by a 5 min final extension at 72 °C. PCR products were monitored in agarose gel with a 1 % ratio. Two-way reading was applied to the amplification products. PCR purification process was realized before sequence analysis. The purification and sequencing process was realized by the Macrogen Company.

Table 3. The base sequences of the primers used (Taberlet *et al.,* 1991).

Primers	Base sequences (5' – 3')
ITS 5 (F):	5' GAA AGT AAA AGT CGT AAC AAG G 3'
ITS 4 (R):	5' TCC TCC GCT TAT TGA TAT GC 3'
trn a (F):	5' CAT TAC AAA TGC GAT GCT CT 3'
trn d (R):	5' GGG GAT AGA GGA CTT GAA C 3'

The obtained data was uploaded to NCBI and GenBank accession numbers were taken. The GenBank accession numbers were given in Table 4.

Phylogenetic Analysis

Phylogenetic analysis was conducted using the program Molecular Evolutionary Genetics Analysis software (MEGA X) (Kumar *et al.,* 2019). In order to evaluate the data of chromatograms (sequencing), Finch TV 1.4 version is used. DNA sequence alignments of 22 individuals, Variable sites, number of parsimony informative sites, genetic distance, nucleotide diversity, and divergence within species were computed by MEGA X version. DNA sequence alignment of all the individuals is made subject to statistical analysis within the scope of this program. Ultimately, phylogenetic trees were constructed by Maximum Parsimony Method with 100 bootstrap replicates (Nei and Kumar, 2000; Kumar *et al.,* 2019).

Table 4. GenBank accession numbers for the rDNA ITS (ITS1-5.8S-ITS2) and *trn*T-*trn*L3' regions of the studied samples

Specimens	GenBank Accesion Numbers	
	ITS region	*trn*T-*trn*L3' region
Artemisia fragrans 1	MT159779	MT648006
Artemisia fragrans 2	MT159780	MT648007
Artemisia fragrans 3	MT159781	MT648008
Artemisia fragrans 4	MT159782	MT648009
Artemisia fragrans 5	MT159783	MT648010
Artemisia fragrans 6	MT159784	MT648011
Artemisia fragrans 7	MT159785	MT648012
Artemisia fragrans 8	MT159786	MT648013
Artemisia fragrans 9	MT159787	MT648014
Artemisia fragrans 10	MT159788	MT648015
Artemisia fragrans 11	MT159789	MT648016
Artemisia fragrans 12	MT159790	MT648017
Artemisia spicigera 1	MT159791	MT648018
Artemisia spicigera 2	MT159792	MT648019
Artemisia spicigera 3	MT159793	MT648020
Artemisia spicigera 4	MT159794	MT648021
Artemisia spicigera 5	MT159795	MT648022
Artemisia spicigera 6	MT159796	MT648023
Artemisia spicigera 7	MT159797	MT648024
Artemisia taurica 1	MT159798	MT648025
Artemisia taurica 2	MT159799	MT648026
Artemisia taurica 3	MT159800	MT648027

RESULTS

Morphological results

It was observed that *A. taurica* and *A. fragrans* species had a larger size compared to *A. spicigera* species in accordance with the environmental conditions it grows and the number of chromosomes and ploidy levels. Detailed morphological measurements of the studied species are as in Table 5.

Karyological results

A. spicigera K.Koch

This taxa general spread is the Eastern Anatolia Region in Turkey. Samples were collected from three populations and 6 individuals of three different localities (Table 1). The samples were labelled "P.Y. 5007-5008-5009-5013-5022-5024". The number of chromosomes in all the samples examined was $2n=2x=18$ and it consists of 4M, 10m and 4sm chromosomes. The metaphase chromosome length is 1.46–3.06 μm and longest to shortest chromosome ratio is 2.0:1. Chromosome arm ratios are 1.28–2.27 μm, the centromeric index is 30.55–43.83 μm, and relative lengths are 4.20–8.78 μm (Table 2, Figure 1,). Secondary structures and satellite chromosomes (sat-chromosome) were not observed in this specimens.

A. taurica Willd.

The species *A. taurica* shows the wide distribution in the steppes of Central, Eastern and Southeastern Anatolia in Turkey. Samples were collected from 6 individuals of three different localities (Table 1). The samples were labelled "P.Y. 5004-5027-5028". The number of chromosomes in all the samples examined was $2n=4x=36$ and it consists of 2M, 14m and 2sm chromosomes. The metaphase chromosome length is 3.40–3.94 μm. Chromosome arm ratios are 1.27–2.57 μm, the centromeric index is 33.09–49.20 μm, and relative lengths are 5.08–5.89 μm (Table 2, Figure 2). Secondary structures and satellite chromosomes (sat-chromosome) were not observed in this specimens.

Table 5. Comparison in terms of key features that distinguish of the species of *A. spicigera*, *A. taurica* and *A. fragrans*

Characters	*A. spicigera*	*A. fragrans*	*A. taurica*
Stem length (cm)	20–50	20-75	20–45(–60)
Dimensions lower leaves (cm)	0.5–2.5 × 0.3–1.5	2.5-4 × 1–2	1–2.5 × 0.5–1.2
Dimensions of cauline leaves (cm)	0.5–1.5 × 0.3–1.5	1-2.5 × 0.5–1.5	0.5–2.5 × 0.3–1
Dimensions of floral leaves (cm)	0.1–1 × 0.1–0.2	0.1–1.2 × 0.1–0.8	0.1–1 × 0.1–0.4
Orientation of synflorescence branches	usually horizontal	usually ascendant	usually horizontal
Capitula length (mm)	1–3 mm long	1–5 mm long	(1–) 3–5 mm long,
Outer phyllaries dimensions (mm)	0.2–0.4 × 0.2–0.4	0.5–0.8 × 0.3–0.5	0.6–0.9 × 0.5–0.8
Middle phyllaries dimensions (mm)	1.2–2.2 × 0.5–1	1–1.2 × 0.8–1.5	1–2.2 × 1.3–1.7
Inner phyllaries dimensions (mm)	3–3.4 × 0.5–1	3.3–3.8 × 1–1.2	4–4.2 × 1.2–1.5
Corolla colour	yellow or red	yellow or red	yellow or pinkish red or purplish red
Corolla dimensions (mm)	2.5–3.2 × 0.5–0.8	1.7–3.5 × 0.2–0.6	2.8–3.3 × 0.5–1
Pistil length (mm)	1.8–3	2.1–3.2	3.1–3.9
Ovarium dimensions (mm)	0.4–0.6 × 0.2–0.4	0.5–0.8 × 0.3–0.6	0.7–1 × 0.2–0.7
Style length (mm)	1.2–1.6	1.5–1.9	1.5–2.2
Forks length of bifid stigma (mm)	0.2–0.5	0.3–0.7	0.4–0.7
Stamens length (mm)	2.2–3.2	3–3.5	3–4.2
Filaments length (mm)	0.8–1.3	1.3–1.6	1–1.5
Anhters dimensions (mm)	1.4–1.7 × 0.1–0.3	2–2.5 × 0.2–0.5	2–2.7 × 0.1–0.3
Number of flowers in capitula	3-5	5-8(-10)	
Achenes (cypselas) dimensions (mm)	1.2–2.2 × 0.5–1.2	2–2.5 × 0.8–1.4	1.8–2.7 × 0.8–1.4
Somatic chromosome number	$2n=2x=18$	$2n=4x=36$	$2n=4x=36$, $2n=6x=54$

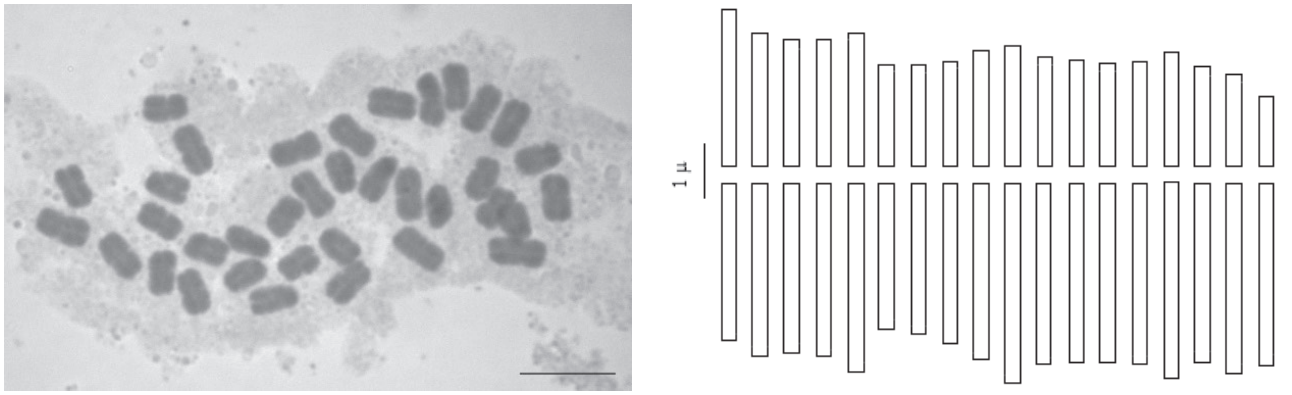

Figure 3. Somatic metaphase in *A. fragrans* ($2n=36$), and haploid idiogram (Scale bars: 1 µm).

A. fragrans Willd.

This taxa only spread is the Eastern Anatolia Region in Turkey and this species a new record for Turkey (Kursat *et al.,* 2014). Samples were collected from five populations and 14 individuals of four different localities (Table 1). The samples were labelled "P.Y. 5001-5002-5003-5005-5006-5010-5011-5012-5016-5017-5019-5020-5021-5023". The number of chromosomes in all the samples examined was $2n=4x=36$ and it consists of 4M, 10m and 4sm chromosomes. The metaphase chromosome length is 1.46–3.06 µm and longest to shortest chromosome ratio is 2.0:1. Chromosome arm ratios are 1.28–2.27 µm, the centromeric index is 30.55–43.83 µm, and relative lengths are 4.20–8.78 µm (Table 2, Figure 3). Secondary structures and satellite chromosomes (sat-chromosome) were not observed in this specimens.

Phylogenetic results

In this part of the study, a phylogenetic tree displaying the phylogenetic position of three *Artemisia* species

Table 6. PCR amplified region length and summary statistics from the rDNA ITS (ITS1-5.8S-ITS2) and the cpDNA (*trn*T-*trn*L3') dataset of genus *Artemisia*.

Molecular Diversity Parameters	ITS (ITS1-5.8S-ITS2) Region	*trn*T-*trn*L3' Region	Co-evaluated of ITS (ITS1-5.8S-ITS2) and *trn*T-*trn*L3' Regions
Total Sample Count	22	22	22
Total Characters	~725	~1020	~1745
GC Ratio (%)	52.7	34.3	41.6
Protected Regions (C)	577	930	1507
Regions with Variation (V)	142	62	204
Parsimony Informative Regions (Pi)	14	20	34

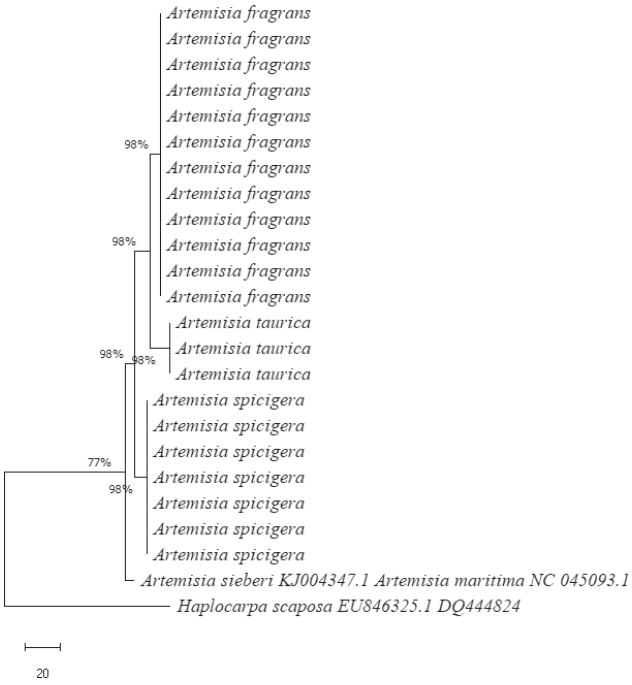

Figure 4. Maximum Parsimony tree obtained from the co-evaluation of sequences of the ITS (ITS1-5.8S-ITS2) and *trn*T-*trn*L3' regions of individuals.

with respect to each other was constructed (Figure 4). Populations of these species were collected from 9 different regions cultivated in Van Lake around and 22 individuals were included in the analyzes. İn addition to, the reference base sequences of two individuals belong to species *A. sieberi* (KJ004347.1) and *A. maritima* (NC045093.1) were also included in our analysis to demonstrate the accuracy of the study (Shahzadi *et.al*., 2020.) *Haplocarpa scaposa* (EU846325.1 and DQ444824) was used as an outgroup (McKenzie et.al. 2006; McKenzie and Barker 2008). Sequence data of plants used as outgroup and sister group were taken from NCBI.

Within the scope of the studies, DNA isolation of 22 individuals from leaf tissue was made by CTAB method, then rDNA ITS (ITS1-5.8S-ITS2) region and non-coding *trn*T-*trn*L3' region of cpDNA was amplified in PCR using specific primers (Table 2). The base sequences of the obtained regions were analyzed and their genetic characteristics were compared and information was obtained about the proximity and distance of taxa to each other. For a more accurate visualization of the results of the alignment, about 50-100 base from the head and the end were not evaluated by us. As a result of the research done from NCBI for *Artemisia* genus, the base length of the ITS (ITS1-5.8S-ITS2) region was found to be 700-750 bp, *trn*T-*trn*L3' in total and the base length was 900-1000 bp in total, and in our study, it was found to be of similar length in accordance with the literature.

The analyses were performed with the X version of the MEGA program and the method that would give the best result for us was selected from the "Find Best DNA Models" step of the program. As a result, it was decided that Maximum Parsimony method would give the most accurate result of the tree drawn with Tamura 3-parameter step. In addition, two different DNA regions were evaluated at the same time and a complex tree has been obtained to achieve a more accurate result (Figure 4).

As a result of the calculations made with the Maximum Parsimony (MP) method, in both the separate and co-evaluations of the sequences of the ITS and trn regions of the examined individuals, in total ~1745 base pairs were taken into consideration and the number of variable regions (V) 204, The number of conserved regions (C) was 1507 parsimony number (PI) 34, and GC ratio was 41.6%. These calculated values are given in Table 6.

DISCUSSION

Artemisia is one of the most complex genera and it is represented by the large number of species, diverse morphological types, ploidy and complicated genetic relationships (Winward and Mcarthur 1995). Because of this, the clarification of the genus's taxonomy using classical botanical tools and morphological characteristics has many difficulties (Torrel *et al.,* 1999). Therefore,

usage of molecular markers and caryological data is a valuable and promising addition to the traditional morphology-based classification (Turuspekov *et al.,* 2018). In this study a phylogenetic systematic study is conducted by using the morphological, karyological and phylogenetic data of three species in *Artemisia* that grow around the Van Lake in Turkey. 22 individuals of taken from 8 different populations belong to taxa of the *A. spicigera*, *A. taurica* and *A. fragrans* were examined morphological measurements, karyotype analysis and analysing the base slice of the regions being obtained, it was tried to get information about the closeness and distance of taxa with each other. This research is important as it is the first molecular based study relating with some *Artemisia* species growing naturally that around the Van Lake in Turkey. Additionally, the first molecular data about these species from Turkey has been submitted to the GenBank international databases.

A lot of research has been carried out to better understand the morphological, karyological, anatomical, and phylogenetic analysis of the genus *Artemisia* and its relationships to the other (four) subgenera, *Absinthium, Dracunculus* (Besser) Rydb, *Seriphidium* Besser ex Less. and *Tridentatae* (Rydb), in different parts of the world. Polyploidy is currently considered a prominent force in plant evolution and represents the most common mode of sympatric speciation in plants (Wendel and Doyle 2005). Polyploids, moreover, may have superior levels of adaptability and higher probabilities of survival than their diploid relatives (Thompson and Lumaret 1992; Soltis and Soltis 2000). Most of the *Artemisia* that colonize extreme and arid habitats are polyploids. This fact supports the hypothesis that polyploids have more tolerance of extreme environmental conditions (Pellicer *et al.,* 2007).

Chromosome data currently available show polyploidy to be the most significant evolutionary trend in chromosome number within Asteraceae (Chehregani *et al.,* 2010). Accordig to Chehregani *et al.* (2010), the highest variation in chromosome number was observed in *A. spicigera*. In this species; different chromosome numbers ($2n=2x=18$, $2n=3x=27$, $2n=4x=36$, $2n=5x=45$, $2n=6x=54$ and $2n= 8x= 72$) were identified in different populations that collected from different parts of Iran. However, in our study, the number of chromosomes in all studied populations was found to be $2n=2x=18$. But Tabur *et al.* (2014), the number of chromosomes in all *A. spicigera* populations they work from Turkey have recorded as $2n=2x=18$. Accordingly, the ploidy level of *A. spicigera* kind in Turkey, we can say that $2x$.

The phylogenetic relationship among the different *Artemisia* species collected from different regions of Pakistan based on the chloroplast gene *RPS*11 was investigated by Mahmood *et al.* (2011). The molecular phylogenetic analyses of the Hawaiian *Artemisia* and its worldwide divergence based on nuclear and chloroplast DNA markers were reported by Hobbs and Baldwin. (2013). As discussed by Haghighi *et al.* (2014), the phylogenetic relationships among *Artemisia* species based on nuclear ITS and chloroplast *psb*A-*trn*H DNA markers using three sections of *Artemisia*, *Dracunculus* and *Serphidium* propose that the ITS and cpDNA *psb*A-*trn*H markers are practicable in the systematic revision of troubled taxa at the intra-genus level in plants. Furthermore, Pellicer *et al.* (2014) performed phylogenetic analysis of the annual *Artemisia* within its major lineages and suggested that annual *Artemisia* have been specially misidentified at a subgeneric level and verified that they are phylogenetically restricted to basal grades. However, to date, very few *Artemisia* species have been verified with molecular phylogenetic studies based on the nucleotide sequence data in Turkey (Koloren *et al.,* 2016).

Civelek *et al.* (2010) have carried out a revisionary study of the genus *Artemisia* in Turkey. According to results of the revisionary study based on the morphological features, it was observed that growing around the Lake Van that in the populations thought to belong to the *A. spicigera* species, there are some groups showing significant morphological differences from this species. These groups were found to be similar to *A. spicigera* and *A. taurica* in terms of morphological, but it has been accepted that they were closer to *A. spicigera*. In these populations, a new variety (*A. spicigera* var. *vanensis*) belonging to the species *A. spicigera* was made, but the variety was not certain as it was not published (nomen nudum). The researchers stated that they are not sure about the accuracy of this systematic arrangement and stated that these populations should be studied in detail. To solve this systematic problems, in Flora of Turkey specified to be very close to each other *A. spicigera* and *A. taurica* species of, planned to investigate detailed morphological and cytogenetic aspects and the research was conducted. While these studies continue, after literature search and cytogenetic observations in these populations, have been identified as belonging to the species *A. fragrans* case a new record for the flora of Turkey and published (Kursat *et al.,* 2014). However, it has been stated that a molecular study is needed to confirm these results. For this purpose, it was decided to phylogenetically evaluate the populations of *A. spicigera*, *A. taurica* and *A. fragrans* species around the Van Lake with various molecular markers. gramer In molecular studies, ITS (ITS1-5.8S-ITS2) in rDNA and *trn* regions in cpDNA were amplified with specific primers and ana-

lyzed with MEGA program. In the phylogenetic family tree created after the analysis, it was observed that the examined individuals of the species in question were completely separated from each other, and the individuals of each species were grouped among themselves. According to these results, it has been determined that there is no gene flow between the populations of these species and they are completely independent from each other. According to the morphological and caryological data, it has been molecularly proven that the populations considered as *A. spicigera* var. *vanensis* (nomen nudum) are correct to be published as *A. fragrans* species.

CONCLUSIONS

According to this study results, it has been found that there is complete speciation genetic isolation mechanism between the species *A. spicigera*, *A. taurica* and *A. fragrans* that inhibit gene flow. Also *A.fragrans* and *A. spicigera* species are very similar to each other in terms of morphological characteristics. However, since populations of the species *A. fragrans* are autopolyploid, the dimensional values of their morphological squares are larger than those of the species *A. spicigera*.

This study is so important as it is the first molecular based study relating with some species of *Artemisia* growing naturally in Turkey.

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