http://dergipark.gov.tr/trkjnat
Trakya University Journal of Natural Sciences, 19(1): 1-10, 2018
ISSN 2147-0294, e-ISSN 2528-9691
DOI: 10.23902/trkjnat.346537

Research Article

INVESTIGATION OF THE GENETIC STRUCTURE OF SOME ANATOLIAN *Achillea* L. (ANTHEMIDEAE, ASTERACEAE) POPULATIONS USING THE ISSR MARKERS

Efkan BAĞDA

Cumhuriyet University, Faculty of Science, Department of Molecular Biology and Genetics, Sivas, TURKEY
e-mail: ebagda@cumhuriyet.edu.tr

Cite this article as:

Bağda E. 2018. Investigation of the Genetic Structure of Some Anatolian *Achillea* L. (Anthemideae, Asteraceae) Populations Using the ISSR Markers. *Trakya Univ J Nat Sci*, 19(1): 1-10, DOI: 10.23902/trkjnat.346537

Received: 25 October 2017, Accepted: 4 December 2017, Online First: 7 December 2017, Published: 15 April 2018

Abstract: Due to high level of hybridization and polyploidy, the perennial and allogamic *Achillea* L. genus with a complex phyletic structure has about 142 species widely distributed over in the Northern Hemisphere. The genus is widely distributed in Turkey with 48 species half of which are endemic. The gene and diversification center of the *Santolinoideae* section including 18 endemic species is thought to be in Anatolia. There exist no comprehensive molecular phylogenetic study on *Achillea* whose morphological revision in Turkey was completed in 2006.

In the study, phylogenetic analyzes were performed using 10 different oligonucleotides for amplification of ISSR bands based on 74 samples of 18 species from 3 sections of *Achillea* genus. All the oligonucleotides analyzed were found to be polymorphic. The total number of loci is 135 and 44 (33%) of them are parsimony informative. Serious topological differences showing that *Achillea* taxa include both monophyletic and polyphyletic lineages were revealed in phylogenetic trees obtained under UPGMA, MP and BI methods. Sections were not clearly separated in trees with clear species separations. The results of UPGMA and MP analyses showed that *A. vermicularis* Trin. was placed as the outgroup while *A. sipirokorensis* Hausskn. & Bornm. and *A. sintenisii* Hub.-Mor. formed the outgroup together in Bayesian Inference analysis (BI). The obtained clusters of PCA based on binary genetic distance values were consistent with the result of BI analysis. Molecular variation analysis showed that almost all of the molecular variation was completely resulted from variations within populations.

Key words: *Achillea*, ISSR, molecular phylogeny, Turkey.

Özet: Çok fazla hibridizasyon ve poliploidi görülmesinden dolayı karmaşık filetik yapıya sahip çok yıllık ve allogamik *Achillea* cinsi Kuzey Yarımküre üzerinde geniş yayılıma sahip 142 kadar türe sahiptir. Bunlardan 48 tanesi Türkiye'de geniş yayılış göstermektedir ve bu 48 türün yarısı endemiktir. 18 endemik türe sahip *Santolinoideae* seksiyonunun gen ve değişim merkezinin Anadolu olduğu düşünülmektedir. Türkiye taksonlarının morfolojik revizyonu 2006 yılında yapılmış olan bu cinsin, kapsamlı bir moleküler filogenetik çalışması halihazırda bulunmamaktadır.

Bu çalışmada, *Achillea* cinsinin 3 seksiyonundan, 18 türe ait 74 örnekle ISSR bantlarının amplifikasyonu için 10 farklı oligonükleotid kullanılarak filogenetik analizler yapılmıştır. Analiz edilen tüm oligonükleotidlerin polimorfik olduğu görülmüştür. Toplam lokus sayısı 135 olup, 44 (%33) tanesi parsinomik olarak bilgi vericidir. UPGMA, MP ve BI filogenetik analiz yöntemleri ile çizilen ağaçlardan *Achillea* taksonlarının monofiletik ve polifiletik olduğunu gösteren ciddi topolojik farklılıklar belirlenmiştir. Tür ayrımlarının olduğu ağaçlarda, seksiyonların net olarak ayrılmadığı belirlenmiştir. UPGMA ve MP analizi sonucunda *A. vermicularis* Trin., Bayesian çıkarsamalı analiz sonucunda ise *A. sipirokorensis* Hausskn. & Bornm. ile *A. sintenisii* Hub.-Mor. türlerinin birlikte dış grup olarak yerleştiği görülmüştür. İkili genetik uzaklık değerlerine dayalı PCA sonuçlarında görülen kümelenmeler Bayesiyan çıkarsamalı analiz sonuçlarıyla uyumlu gerçekleşmiştir. Moleküler varyasyon analiz sonuçları moleküler varyasyonun tamamına yakınının populasyonlar içinden kaynaklandığını göstermiştir.

Introduction

Turkey has a rich flora because of its geological features, soil types and climate conditions in addition to the fact that it is located at the intersection point of Asian, European and African continents. It has a moving geological structure formed by the closure of the Tethyan Sea and it played an important role during the glacial periods The presence of charecteristics of the Mediterranean, Euro-Siberian and Irano-Turanian plant geographical regions in the country is the most important factor increasing species diversity. The flora of Turkey includes about 9222 vascular plant species of which 138 are cultured. Turkey is also an important gene centre

(%33.27 with 2991 species) with a high endemism rate (Arabacı 2006). However, despite the floral richness and high endemism rate in the country thenumber of molecular phylogenetic studies on floral members is limited.

The first work on Turkey's flora is "Flora Orientalis" written by Geneva's famous botanist Edmond Boissier in 1867-1888, and the most comprehensive work, the book "Flora of Turkey and the East Aegean Islands", was written by P. H. Davis. This book consists of 10 volumes together with additional volume. A second additional volume with an increase in Turkish flora studies was also added to this book (Güner *et al*. 2000).

Studies on Anatolian flora have gained a pronounced acceleration recently and new taxa have been identified and/or the available taxonomic groups have been redesigned in studies carried out with numerous samples collected during intensive floristic studies. Revision studies have been increasingly carried out, particularly at genus level, to solve the existing taxonomic problems. The identification of new taxa, the determination of species boundaries and the rewriting of species keys are important consequences of these studies. On the other hand, complex phyletic relationships that are frequently encountered, especially due to high hybridization and polyploidy rates weaken the solving power of morphological revision studies. Molecular systematic approaches are often preferred to overcome these problems with the special aim of revealing evolutionary relationships.

Members of the family Asteraceae (Compositae) are composed of 24080 species distributed in 1545 genera belonging to 21 tribes and three subfamilies. Most of the species in the family are members of the subfamily Asteroideae in which 12 tribes, 1176 genera and about 17025 species are gathered (Arabacı 2006). In the Flora of Turkey, Asteraceae is represented by 11 tribes, 136 genera and 1195 species and is the richest family of flora in terms of both species and genus levels. The family also includes most of the endemic species (endemism rate is %37.3 with 446 species) of the country (Arabacı 2006).

The genus *Achillea* L. is represented with 142 species from the Anthemideae tribe of Asteroideae subfamily and is one of the most recently evolved genus of the family (Arabacı 2006, Rahimmalek *et al.* 2009). Members of the genus can grow in almost all habitat types, from the sea level up to altitudes of 3000m a.s.l., mainly in the temperate zone. It is characterized by perennial and allogamic plants adapted to various ecological environments ranging from deserts to sea shores, steady snowy hills and rocky habitats. (Guo *et al.* 2004). The genus is widely distributed in Europe and West Asia, but it is represented with several species in North America, Australia, New Zealand and North Africa (Rechinger 1963).

The total number of species of *Achillea* in Turkey is 48 (58 taxa) of which 25 are endemic for the country (Ehrendorfer & Guo 2005, Arabacı 2006, Çelik & Akpulat 2008, Arabacı & Budak 2009, Arabacı 2012, Aytaç *et al.* 2016), indicating the high endemism rate in Turkey (Arabacı 2006).

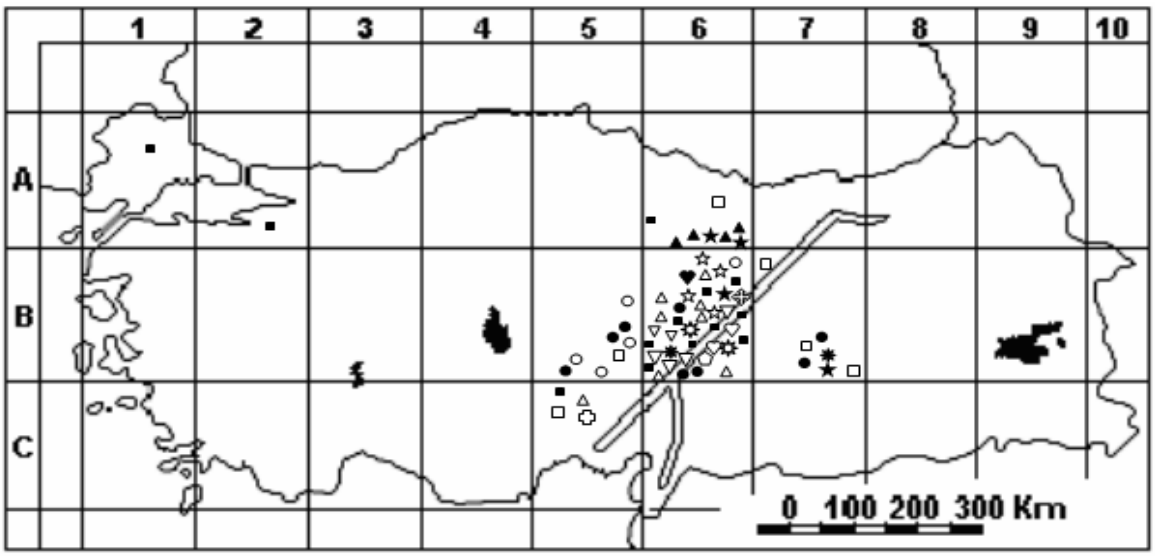
It is thought that the gene center of *Achillea* genus may be the area of the Asian and European continents coalesce. However, the *Santolinoideae* (DC.) Heimerl section of 38 varieties is represented by 26 species, 18 of which are endemic in Turkey, indicating that the genetic and diversification center of *Santolinoideae* section is in Anatolia (Ehrendorfer & Guo 2006, Aytaç *et al.* 2016). In addition, ITS and *trn*L-F analysis suggest that the ancestral section may be *Santolinoideae* based on the results of Guo *et al.* (2004). It has been reported by Arabacı (2006) that the *Achillea* species are mostly concentrated in the Anatolian diagonal, showing that the Anatolian diagonal is an important area in the evolution of the *Achillea* genus in Anatolia.

The phylogenetic relationships and various polyploidy species models in *Achillea* species are investigated with nuclear ribosomal ITS, ncp*GS*, *Pgi*C, *SBP*, chloroplast *trn*T, *trn*F, *trn*H-*psb*A, *trn*C-*ycf*6, *rp*L16 genes with the markers of isozyme electrophoresis, RAPD, AFLP, RFLP, microsatellite and ISSR (Purdy & Bayer 1996, Guo *et al.* 2004, Ehrendorfer & Guo 2005, Guo *et al.* 2005, Ehrendorfer & Guo 2006, Guo *et al.* 2006, Guo *et al.* 2008, Morsy 2007, Rahimmalek *et al.* 2009, Ma *et al.* 2010, Gharibi *et al.* 2011, Rawashdeh 2011, Rahimmalek *et al.* 2011, Ebrahimi *et al.* 2012 a b, Farajpour *et al.* 2012, Guo *et al.* 2012, Rahimmalek 2012, Badr *et al.* 2014, López-Vinyallonga *et al.* 2015, Inotai *et al.* 2016, Badr *et al.* 2017).

High biodiversity and natural hybrids in *Achillea* make it difficult to identify plant samples. Molecular markers are powerful alternative solutions especially for systematic problems which cannot be solved by morphological approaches. In this study, we aimed to contribute to the information about the genus systematic by investigating the biogenetic loci and genetic structures and phylogenetic relationships of 18 cultivars (10 endemic) of *Achillea* L (Asteraceae) genus spreading in and around Sivas using ISSR markers (Zietkiewicz 1994).

Materials and Methods

18 *Achillea* species collected during the period from 2011 to 2012 were used as the study material (Fig. 1, Table 1). All collected specimens are kept in the herbarium of the Cumhuriyet University Faculty of Science (CÜFH). Air dried samples were used for DNA isolation. The total DNA isolations were done using 74 different specimens of 18 species distributed in 59 populations and ISSR-PCR was run for all samples.



A. armenorum (⬠), *A. biebersteinii* (□),*A. cappadocica* (▼), *A. coarctata* (○), *A. cucullata* (✲), *A. kotschyi* (⯐), *A. lycaonica* (☆), *A. magnifica* (✸), *A. millefolium* (■), *A. nobilis* (●), *A. phyrgia* (✥), *A. schischkinii* (★), *A. sipikorensis* (♡), *A. sintenisii* (▲), *A. sivasica* (♥), *A. teretifolia* (▽), *A. wilhelmsii* (△), *A. vermicularis* (⊕)

Fig. 1. Distribution map of *Achillea* populations used in the study.

Table 1. Herbarium numbers (Hrb. No.) and localities of *Achillea* specimens used in the study.

No	Species	Hrb.No	Abbr.	Square	Localities
1	*A. armenorum* Boiss. & Hausskn.	15330	arm	C6	Kahramanmaraş: Göksun, Berit Mountain
2	*A. biebersteinii* Afan	15283	bieb 1	A6	Ordu: Mesudiye-Koyulhisar highway
3	*A. biebersteinii*	15293	bieb 2	B6	Sivas: Zara-Divriği highway, Çaypınar Village
4	*A. biebersteinii*	15367	bieb 3	B9	Between Tatvan-Gevas
5	*A. biebersteinii*	15309	bieb 4	B7	Malatya: Airport highway, Aksaray Village
6	*A. biebersteinii*	15370	bieb5	B5	Sarıkaya-Yozgat
7	*A. biebersteinii*	15373	bieb6	B6	Between Göksun- Kahramanmaraş
8	*A. biebersteinii*	15368	bieb 7	B9	7km to Bitlis
9	*A. cappadocica* Hausskn. & Bornm.	15201	cap1	B6	Yozgat, Between Çat-Güzelyayla
10	*A. cappadocica*	15204	cap2	B6	Yozgat, near Kızılcaova
11	*A. cappadocica*	15208	cap3	B6	Yozgat, near Bozhüyük
12	*A. coarctata* Poir	15295	coa 1	B6	Sivas: Zara-Divriği highway, Çaypınar Village
13	*A. coarctata*	15298	coa 2	B5	Kayseri: Hacılar-Develi highway, Erciyes Mountain
14	*A. coarctata*	15299	coa 3	B5	Kayseri: Sivas-Kayseri highway
15	*A. coarctata*	15304	coa 4	B5	Kayseri: Develi-Bakırdağı, Şahmelik Village
16	*A. coarctata*	15307	coa 5	B5	Kayseri: Hacılar-Develi highway, Hacılar out way
17	*A. cucullata* (Hausskn.) Bornm.	15226	cuc 1	B6	Sivas: Taşlıdere
18	*A. cucullata*	15241	cuc 2	B6	Sivas: Karaçayır highway
19	*A. kotschyi* Boiss.	15345	kot1	C5	Adana-Niğde highway, Niğde Entrance
20	*A. kotschyi*	15207	kot2	B6	Yozgat: Çayıralan out way
21	*A. lycaonica* Boiss. & Heldr.	15151	lyc 1	B6	Sivas: Ulaş, Tecer-Eskikarahisar Village
22	*A. lycaonica*	15153	lyc 2	B6	Sivas: Ulaş, Bostankaya Village
23	*A. lycaonica*	15354	lyc 3	B6	Sivas, Ulaş, Hacımirza Village
24	*A. lycaonica*	15262	lyc 4	B6	Sivas: Cemel-Altınyayla highway
25	*A. magnifica* Hub.-Mor.	15181	mag 1	B6	Sivas: Divriği-İliç highway, Gedikbaşı 8km
26	*A. magnifica*	15310	mag 2	B7	Malatya: arround airport
27	*A. millefolium* L.	15235	mil 1	B6	Sivas: Zara, Karabayır

No	Species	Hrb.No	Abbr.	Square	Localities
28	*A. millefolium*	15325	mil 2	B6	Kahramanmaraş: Göksun, Mehmetbey Village
29	*A. millefolium*	15340	mil 3	C5	Niğde: Çamardı-Yeniköy highway
30	*A. millefolium*	15365	mil 4	B9	Between Tatvan-Gevaş, 63km to Gevaş
31	*A. millefolium*	15275	mil 5	A6	Tokat: Çamlıbel, İhsaniye Village
32	*A. millefolium*	15321	mil 6	B6	Kayseri: Bünyan-Pınarbaşı highway, Erkek Village
33	*A. nobilis* L.	15215	nob 1	B6	Sivas: Ulaş, Hüyüktepe south hillside
34	*A. nobilis*	15270	nob 2	B7	Malatya: Arapkir-Kemaliye highway, 3km
35	*A. nobilis*	15324	nob 3	B5	Kayseri: Kayseri-Sarız, Sarız entrance
36	*A. nobilis*	15326	nob 4	B6	Kahramanmaraş: Mehmetbey Village
37	*A. phyrgia* Boiss. & Balansa	15164	phy	B6	Sivas: Between Gürün-Kangal, Kuşkayası
38	*A. schischkinii* Sosn.	15228	sch 1	A6	Sivas: Between Suşehri-Şerefiye, Karabayır
39	*A. schischkinii*	15146	sch 2	B6	Sivas: Hafik highway, Soğuk Çermik entrance
40	*A. schischkinii*	15279	sch 3	A6	Sivas: İmranlı-Karacaören, Bahadun detour
41	*A. schischkinii*	15274	sch 4	B7	Malatya: Arapgir-Kemaliye, 20km to Kemaliye
42	*A. sintenisii* Hub.-Mor	15154	sin 1	A6	Sivas: Ulaş, arround Bostankaya Village
43	*A. sintenisii*	15155	sin 2	A6	Sivas: Hafik highway, Soğuk Çermik detour
44	*A. sintenisii*	15187	sin 3	A6	Sivas: Between Hafik-Zara, Topçuyeniköy detour
45	*A. sintenisii*	15282	sin 4	A6	Sivas: İmranlı-Karacaören, Bahadun detour
46	*A. sipikorensis* Hausskn. & Bornm	15281	sip 1	B6	Sivas: İmranlı-Karacaören, Bahadun detour
47	*A. sipikorensis*	15268	sip 2	B6	Sivas: Çetinkaya-Divriği Çetinkaya detour
48	*A. sivasica* Çelik & Akpulat	15163	siv	B6	Sivas: Ulaş, Kovalı Village, arround Ziyarettepe
49	*A. teretifolia*	15236	ter 1	B6	Sivas: Ulaş, Baharözü, Düğnükkaya hill
50	*A. teretifolia*	15267	ter 2	B6	Sivas: Kangal, Höbek Village
51	*A. teretifolia*	15196	ter 3	B6	Sivas: Divriği-Gedikbaşı, Çayözü detour
52	*A. teretifolia*	15245	ter 4	B6	Sivas: Between Şarkışla-Altınyayla, Konakyazı Vill.
53	*A. teretifolia*	15364	ter 5	C3	Antalya-Elmalı-Güğübeli
54	*A vermicularis* Trin.	15369	ver	B9	Hakkari-Van detour
55	*A. wilhelmsii* C. Koch.	15162	wil 1	B6	Sivas: Ulaş, Kovalı Village
56	*A. wilhelmsii*	15261	wil 2	B6	Kayseri: Kaftangiyen-Taşlıgeçit Village
57	*A. wilhelmsii*	15344	wil 3	C5	Niğde: Maden Village
58	*A. wilhelmsii*	15220	wil 4	B6	Sivas: Kangal-Kazıklı bridge
59	*A. wilhelmsii*	15287	wil 5	B6	Sivas: İmranlı-Karacaören Village

Total Genomic DNA Isolation

Total genomic DNA isolations were done in equal amounts of tissue samples by modifying the CTAB procedure described by Doyle & Doyle (1987). Care was taken to select leaf samples whenever possible. 694 DNA isolations were performed in total from 294 different individuals. DNA samples were stored at +4°C by dissolving in 100µl 1xTE (10mM Tris-HCl, 1mM EDTA, pH 8.0). Total genomic DNA samples were checked by loading on 1% agarose gel.

Determination of Quality and Quantity of Genomic DNA

The quality and quantitation of DNA after isolation were determined by both agarose gel (1%) electrophoresis technique and spectrophotometric measurements at 260 and 280nm wavelengths. The quality and quantity of the DNA samples were estimated by electrophoresis band pattern and by comparing with DNA marker.

Amplification of ISSR Fragment by PCR

ISSR fragments were amplified by PCR using 10 different oligonucleotides (Table 2).

ISSR fragments, 4ng/µL of template DNA (100ng/µL), 1x *Taq* buffer [10x*Taq* Buffer; 100mM Tris-HCl (pH 8.8), 500mM KCl, 0.8% Nonidet P40], 1.5mM $MgCl_2$ (25mM), 0.1mM dNTP mix (each dATP, dTTP, dCTP, dGTP 0.5mM), 0.02U/µL *Taq* polimeraz (5U/µL), 0.2pmol/µL primer (25pmol/µL) were diluted to 25 µL with sterile distilled water and then amplified by PCR. For amplification; 94°C for 30sec, 65°C (Table 2) for 1min and 72°C for 1 min PCR temperature profile was applied over 35 cycles. PCR products were checked by loading on 1.5% agarose gel (Fig. 2).

Analysis of ISSR Data

The phylogenies of *Achillea* populations were investigated using different algorithms. Analyzes were

performed on three different approaches; DNA distance, maximum parsimony (MP) and Bayesian inference. Analyzes were evaluated according to the band profiles obtained from the ISSR markers. The presence of the bands is indicated by "1" and the absence of bands by "0". The presence of the band represents a dominant, non-existent recessive phenotype. Because ISSR markers are dominant, the genotype and allele frequencies can not be calculated since the alleles in the same locus can not be distinguished. Therefore, ISSR data is calculated based on the ratio of bands that are common to any locust to all bands. The ratios of existing bands (1) or non-existing bands (0) and common bands were used for the calculations. All these calculations are based on the assumption that the bands moving in the gel for the same distance (R_f), that is, of the same size, are similar. In fact, bands of similar length are directly proportional to the kinship grades of the compared individuals. So, we can say that individuals with more common bands are closer and those with less common bands are farther away. The obtained trees were drawn using FigTree v1.3.1 (Rambaut 2009).

Analysis of the obtained data was performed using a computer program called Popgene 32 (version 1.3.1) (Yeh *et al.* 1999). Assuming that the populations were in the Hardy-Weinberg equilibrium, the genetic distance (Fst) values between population pairs were calculated to determine population differentiation (Nei 1972).

The unweighted pair-group method with arithmetic mean (UPGMA) dendograms (10000 replicates) were generated by varying the Fst values of the Neighbor-Joining (Saitou & Nei 1987) procedure using the same analysis program. PCA (Principal Component Analysis) was used in the GenAlEx 6.3 package program (Peakall & Smouse 2006) to create a visual representation of the genetic relationship between populations.

Hierarchical analysis of the molecular variation (AMOVA) in the genetic construction of *Achillea* populations was performed using the Arlequin 3.11 (Excoffier *et al.* 2005) program using clusters obtained from biogeographic areas, phylogenetic and principal component analyzes. The significance ratings of fixation indices determined by AMOVA were determined by testing with 1000 proposed permutations given by Excoffier *et al.* (1992).

The MP analysis of the data sets was performed by applying the tree bisection-reconnection (TBR) branch swapping, random addition sequence replicates and 50% majority rule using PAUP * 4.0 beta 10 (Swofford 2002) with heuristic search algorithm. Character states were unordered and unweighted. The bootstrap values of the branches were investigated using heuristic search with 1000 bootstrap replicates.

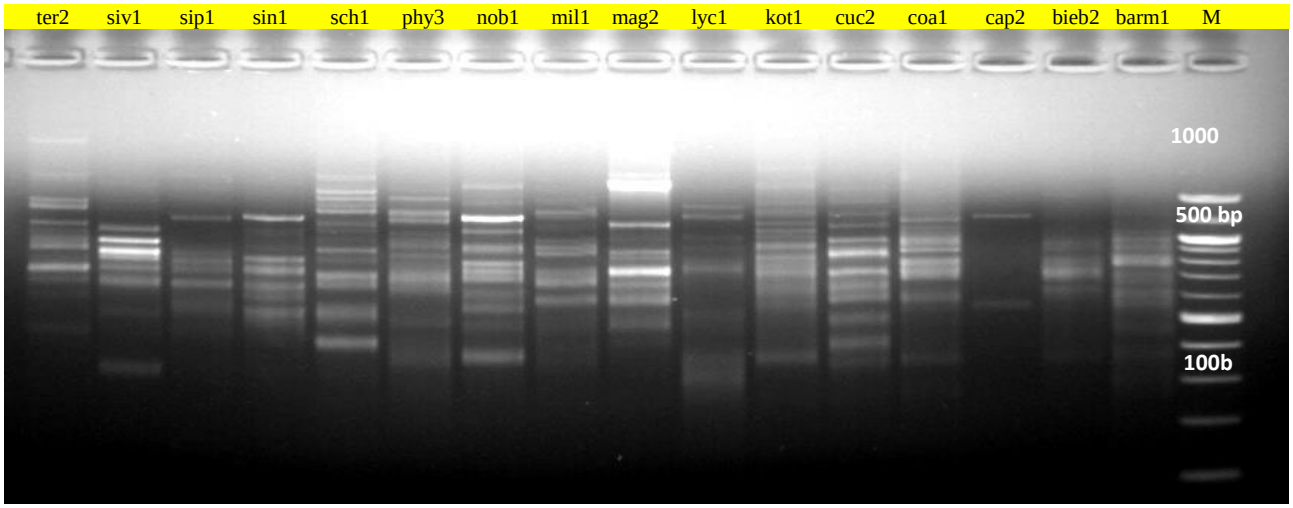


Fig. 2. DNA fragments amplified with ISSR-1 oligonucleotide. Marker (M): 100bp.

Table 2. Base sequences and annealing temperatures of the ISSR oligonucleotides used for amplification.

Name	Sequence	Repeat sequence	Anneling temperature
ISSR 1	CTCTCTCTCTCTCTCTG	$(CT)_8G$	53°C
ISSR 2	CACACACACACACACAG	$(CA)_8G$	53°C
ISSR 3	GAGAGAGAGAGAGAGAG	$(GA)_8G$	53°C
ISSR 4	GTGTGTGTGTGTGTGTC	$(GT)_8C$	58°C
ISSR 5	GTGTGTGTGTGTGTGTC	$(GT)_8C$	51°C
ISSR 6	CACACACACACACACAGAC	$(CA)_8GAC$	57°C
ISSR 7	GAGTCTCTCTCTCTCTCTC	$GAG(TC)_8$	57°C
ISSR 8	CACCACCACCACCACCACCACT	$(CAC)_7T$	61-67°C
ISSR 9	GTCACCACCACCACCACCACCAC	$(CAC)_7GT$	67°C
ISRR 10	TCTTCTTCTTCTTCTTCT	$(TCT)_6$	50°C

In MP analysis, *A. vermicularis* population was determined as the outgroup from UPGMA dendogram and consensus tree was formed. In this way, the most reliable tree was obtained by re-establishing the branches in the consensus tree. Genetic distance and parsinomic results were compared and common and non-common points were evaluated.

Phylogenetic analyses based on Bayesian and Markov Chain Monte Carlo (MCMC) were carried out using the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Analyzes were performed by presence or absence of markers in the populations (field analysis). In the ISSR analysis, "nst=1" and the rate is "rates=equal" was used. For the generation of 10^7, two independent runs of four chains (3 heated and 1 cold chain) were carried out and the trees were sampled every 1000 cycles. Convergence on stationary distribution was verified by checking whether the mean standard deviation of the separation frequencies is less than 0.05 between two independent executions. Bayesian posterior probabilities were estimated by constructing a Majority-Rule Consensus Tree among the last 750 sampled trees (25% of the samples, ie, 250 samples were pre-tested or burn-in removed).

Results

Amplifications of ISSR markers were performed using 10 oligonucleotides from 74 samples of 18 species distributed in 3 sections of the *Achillea* genus (Table 3).

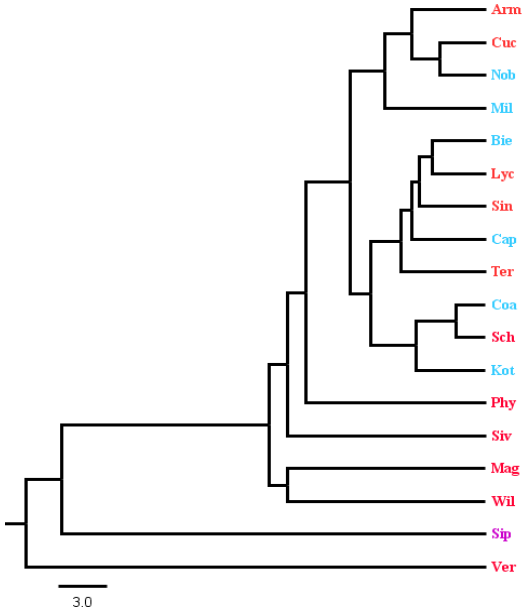


Fig. 3. UPGMA dendogram based on genetic distance data matrix between populations.

All of the oligonucleotides analyzed were found to be polymorphic. The total number of loci was 135 (at most 24, at least 9 bands) and it was determined that 50 of them were fixed, 41 of them were not informative and 44 of them were parsinomically informative.

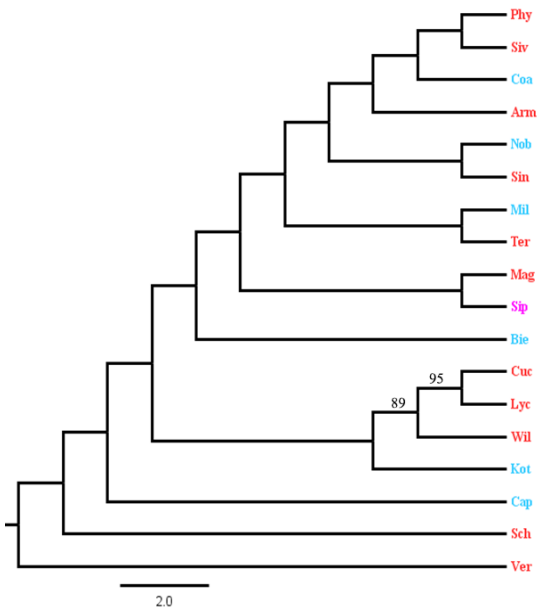


Fig. 4. Compatibility tree with 50% majority rule based on MP method. All branches except two branches (95% and 89%) were shown to support 100%. Colored groups stands for sections represented by populations.

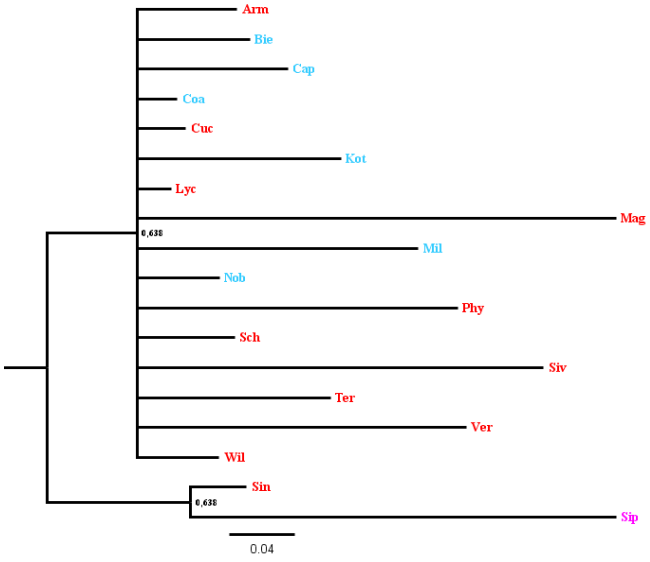
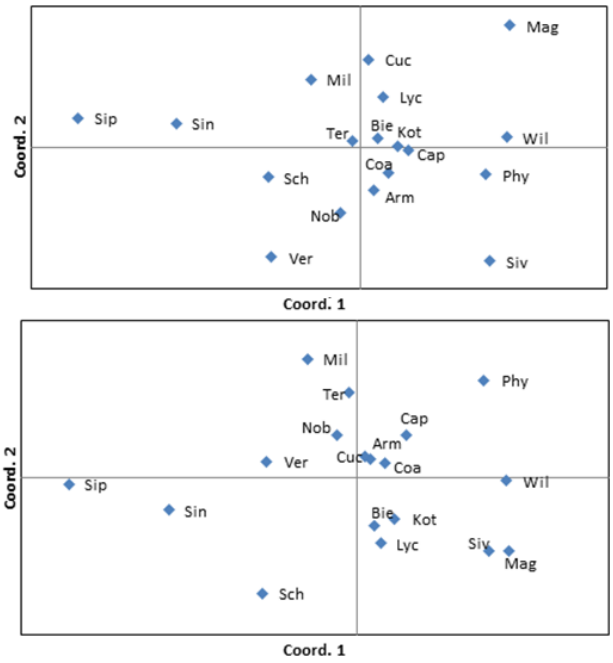


Fig. 5. Phylogenetic relationship between populations based on ISSR haplotypes. The cladogram is a majority-based compliance tree based on Bayesian analysis.

The smallest (0.0676) genetic distance was found between *A. schischkinii* and *A. coarctata* populations and the largest genetic distance (0.8149) was found (Nei 1972) between *A. sipikorensis* and *A. wilhelmsii* populations (Table 4).

It was determined from the UPGMA dendograms based on the genetic distance values between the populations (Fig. 3) that the Anatolian *Achillea* populations were monophyletic and *A. sipikorensis* was the sister group and *A. vermicularis* was the outgroup.



Percentage of variation explained by the first 3 axes			
Axes	**1**	**2**	**3**
%	21.62	19.92	17.17
Cum%	21.62	41.54	58.70

Fig. 6. PCA (Principal Component Analysis) results for the formation of phylogenetic relations between *Achillea* populations. A) Axes 1-2, b) Axes 2-3.

Maximum parsinomy analysis of *A. vermicularis*, which was located as an outgroup on the UPGMA dendogram, was assigned as an outgroup for 50% majority rule application. A consensus tree was constructed with 50% majority rule (CI 0.659; RI 0.476) (see Fig. 4) by using the most suitable 38 consecutive trees of 129 trees. Except two branches (95% and 89%), all branches were found to support 100%. It is found that, as in the UPGMA dendogram, *Achillea* populations are monophyletic in cladogram where *A. schischkinii* is a sister group. The branch settlements were very different and they were not similar except that they were monophyletic in both trees.

Table 3. The number of samples for which ISSR fragments were amplified.

No	Species	Number of Specimens
1	*A. armenorum*	3
2	*A. biebersteinii*	7
3	*A. cappodocica*	3
4	*A. coarctata*	5
5	*A. cucullata*	3
6	*A. kotschyi*	3
7	*A. lycaonica*	5
8	*A. magnifica*	4
9	*A. millefolium*	6
10	*A. nobilis*	4
11	*A. phyrgia*	3
12	*A. schischkinii*	4
13	*A. sintenisii*	4
14	*A. sipikorensis*	3
15	*A. sivasica*	3
16	*A. teretifolia*	6
17	*A. vermicularis*	3
18	*A. wilhelmsii*	5
	Total	74

Table 4. Genetic distance data matrix between populations (Nei 1972).

	Arm	Bie	Cap	Coa	Cuc	Kot	Lyc	Mag	Mil	Nob	Phy	Sch	Sin	Sip	Siv	Ter	Ver	Wil
Arm	0																	
Bie	0.2111	0																
Cap	0.1944	0.1094	0															
Coa	0.1394	0.1693	0.1485	0														
Cuc	0.123	0.1073	0.1434	0.1993	0													
Kot	0.1988	0.2104	0.2099	0.113	0.2248	0												
Lyc	0.1948	0.0861	0.1121	0.1415	0.1007	0.1447	0											
Mag	0.3134	0.2317	0.2392	0.2394	0.2583	0.293	0.2011	0										
Mil	0.2107	0.1896	0.1954	0.2684	0.0986	0.3329	0.2089	0.2854	0									
Nob	0.1132	0.1354	0.1491	0.1916	0.083	0.2676	0.1686	0.3101	0.1401	0								
Phy	0.1474	0.2961	0.3013	0.1845	0.2038	0.2451	0.2872	0.367	0.2147	0.2099	0							
Sch	0.167	0.159	0.161	**0.0676**	0.1953	0.118	0.1282	0.2815	0.2756	0.217	0.2674	0						
Sin	0.2262	0.1104	0.1258	0.1503	0.1545	0.2033	0.1045	0.2455	0.2188	0.1478	0.3343	0.1519	0					
Sip	0.3557	0.6165	0.607	0.4939	0.4718	0.4451	0.5856	0.6635	0.4848	0.4757	0.46	0.4908	0.5215	0				
Siv	0.256	0.2483	0.2481	0.3204	0.1725	0.3954	0.263	0.3239	0.2117	0.1854	0.2843	0.3296	0.2901	0.5196	0			
Ter	0.2206	0.1137	0.1471	0.1629	0.1519	0.2199	0.1322	0.2367	0.1993	0.173	0.2861	0.1753	0.122	0.597	0.2872	0		
Ver	0.6552	0.5948	0.6282	0.5739	0.6906	0.6407	0.603	0.644	0.5691	0.6143	0.5316	0.5167	0.5528	0.6739	0.5798	0.516	0	
Wil	0.4125	0.2383	0.3012	0.3146	0.3012	0.3677	0.2309	0.2624	0.2716	0.3522	0.2983	0.3204	0.2691	**0.8149**	0.3299	0.2407	0.3466	0

The population was found to be polyphyletic from the trees formed by Bayesian Inference analysis based on haplotype distribution. In addition, unlike the others, *A. sipikorensis* and *A. sintenisii*, which are monophyletic among themselves, co-existed as outgroups together (Fig. 5).

Molecular variation in the genetic construct (AMOVA) for *Achillea* population was carried out using clusters obtained from phylogenetic and basic component analyzes, as well as some biogeographic fields (Anatolian Diagonal considered). It has been shown that almost 100% of the molecular variation originated from within the populations (no results were given).

Two different clusters were observed according to PCA based on binary genetic distance values (Fig. 6). The first two axes of the major components bring out 51.16%

of the total genetic variation. The total variation ratio of the primary components to the first and third axes is 48.68%, and the total variation ratio of the axes 2 and 3 is 40.81%. While *A. sipikorensis* and *A. sintenisii* were located differently in both distributions, no significant distribution was observed in other populations. This distribution model is found compatible with the results of Bayesian analysis.

Discussion and Conclusion

Determination of the phylogenetic relationships of the genus *Achillea*, which has a complex phyletic structure due to hybrid and polyploidy frequency is problematic (Guo *et al.* 2004, Guo *et al.* 2012). Morphological revision of Turkey's *Achillea* taxa was conducted by Arabacı (2006) but molecular phylogenetic studies on the genus are limited and they were mostly based on inadequate sample size. Therefore, the present study is the most comprehensive molecular phylogenetic study so far on Anatolian *Achillea* species and aimed to contribute to the systematic information using ISSR markers of 18 species from three sections.

Turkey is an important evolutionary unit for the genus *Achillea* (ESU) considering the fact that 46 species of *Achillea* which constitute 1/3 of the total number of the species within the genus are found in Turkey and half of these species are endemic. Due to this evolutionary importance, species diversity needs to be well investigated. The *Santolinoideae* section has 38 species. Of these, 16 are endemic and 24 are located in Turkey. This indicates that the gene and the center of change of the *Santolinoideae* section is Anatolia Guo *et al.* (2004) suggested based on the results of ITS and *trn*L-F sequence analysis that the ancestral section may be *Santolinoideae*, providing an interesting detail for the origin of the genus. *A. teretifolia* and *A. wilhelmsii*, both belonging to the *Santolinoideae* section, were located in the ancestral clades in the study of Guo *et al.* (2004). The ISSR data obtained in the present study supports the *Santolinoideae* section as an outgroup, but *A. teretifolia* and *A. wilhelmsii* species were not found in the outgroup. UPGMA and MP support *A. vermicularis* (*Santolinoideae*) as an ancestral taxon, while BI results support the external group as *A. sintenisii* (*Santolinoideae*) and *A. sipikorensis* (*Arthrolepis*). It should be mentioned that, all sections are not included in the present study.

The phylogenetic tree patterns from the phylogenetic analysis of *Achillea* populations containing a large number of hybrids and polyploidy species/taxa are one of the most likely scenarios to be expected and this pattern has also been obtained from MP and BI cladograms generated from ISSR data. The data given by Guo *et al.* (2004) also supports a similar polyphyletic story based on analysis of ITS and *trn*L-F sequence data. Significant major differences were determined between the results of the alternative analysis, but *Achillea* populations were considered to be monophyletic by the analysis (without the choice of outgroup).

Some of the results obtained from the analysis were found to overlap with some information given in the Anatolian revision study of Arabacı (2006). *A. armenorum*, an endemic species unique to the Berit Mountain, has distributed in rocky areas above 2400m. There is no *Achillea* species that are similar or closely related to *A. armenorum* (Arabacı 2006).

BI cladogram and PCA, the pattern in which the monophyletic *A. sintenisii* and *A. sipikorensis* were evaluated together as an outgroup support the finding that two revised polyploidies are given in the revision.

A. sintenisii and *A. sipikorensis* which grow between 1200-2000m on gypsiferous peaks are endemic to Irano-Turanian region. They are distributed intensively in steppe and calcareous slopes in Sivas and its vicinity. The species commonly found in gypseous areas are close relatives and the spreading areas are the same or close together. There are significant differences between the populations of *A. sipikorensis* on the gypsifer bed rock and others (Arabacı 2006).

The ISSR data obtained from the study did not fully support the hypothesis that Anatolian Diagonal may have played an important role in the evolution of Anatolian *Achillea* populations. In addition to the sympatric speciation expected to be effective in the evolution of the *Achillea* taxa in Anatolia, the vicariance speciation model needs to be tested again using more extensive samplings. Thereby, looking at the nucleotide sequence variations, AFPL marker or chloroplast will increase the reliability of the result.

The present study is the first study on molecular phylogeny of non-endemic (*A. schischkinii, A. vermicularis*) and endemic (*A. armenorum, A. cappadocica, A. cucullata, A. kotschyi, A. lycaonica, A. magnifica, A. phyrgia, A. sintenisii, A. sipikorensis, A. sivasica*) *Achillea* species in Turkey. In addition, to the best of our knowledge, ISSR markers of 17 species (except *A. millefolium*) included in the study were obtained for the first time.

In conclusion, ISSR markers provided a comprehensive and significant contribution to the understanding of the genetic diversity and phylogenetic relationship of *Achillea* taxa in Turkey. The results obtained will help to understand the evolutionary dynamics of *Achillea* genus.

Acknowledgement

This study is financially supported by the Scientific Research Project Fund of Cumhuriyet University (CÜBAP) under the project number F-405. The author thanks to CÜTAM (Cumhuriyet University Advanced Technology Research Center), to Nuray Akkaya Zonuz for collecting the specimens and allowing the use of material for the study and to Erol Dönmez who described the species and prepared the herbarium specimens.

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