Molecular phylogeny based on its sequences of nrDNA ITS of *Adonis* (Linnaeus, 1753) (Ranunculaceae) from various ecological sites of Turkey

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Abstract

Background Genus *Adonis* (Linnaeus, 1753) contains approximately 40 annual and perennial species, which are widely distributed in the temperate zones of Asia and Europe, and less frequently in southwestern Asia, northern Africa and the Mediterranean region. In the present study, molecular phylogeny of genus *Adonis* L. from Turkey was evaluated with the aid of nrDNA ITS.

Methods Samples of 10 natural *Adonis* taxa belonging to 21 different populations including 64 *Adonis* ecotypes were collected from different regions of the country during vegetation period between 2014 and 2018. ITS1, ITS4, P16 and P25 primers within Internal Transcribed Spacer (ITS) technique were used to estimate infraspecific variation in different populations of *Adonis* species.

Results We identified about 600 bp DNA sequences were obtained from 21 different populations including 64 *Adonis* ecotypes. The dendrogram obtained from *Adonis* species and out-group sequences had two large main groups. While the out-group species were placed in the first large main group, the sect. *Consiligo* (perennial) and sect. *Adonis* (annuals) were placed in different sub-groups of the second large main group. Genetic similarity among *Adonis* species varied between *A. microcarpa* and *A. dentata* (98.46%).

Conclusions Present analyses revealed that phylogenetic classification (grouping) of *Adonis* taxa largely depended on morphological structure and present ITS primers were quite efficient in putting forth the genetic diversity of such species. The results obtained from molecular data can be used to explore the genetic variation pattern, population structure, and the evolutionary history of natural *Adonis* species in the future.

Introduction

Ranunculaceae family, distributed almost all over the world, is considered as one of the essential groups of ancient angiosperms. It is a monophyletic family and estimated to have a history of approximately 75 million years according to the fossil records [1–3]. This family with about 43 genera and 2346 species is represented by 20 genera and 204 species in Turkey and 51 of these species are endemic [4, 5].

Within the family, *Adonis* L. genus contains approximately 40 annual and perennial species, which are widely distributed in the temperate zones of Asia and Europe, and less frequently in southwestern Asia, northern Africa and the Mediterranean region [6–11]. *Adonis* species are distributed in habitats such as fallow fields, cultivated fields, industrial areas, airports, railway, highway sides, mountain steppes, forest, meadows, rocky slopes, shrubs, ruins, pastures and altitudes between 0-3500 m. [12]. In the latest taxonomic studies, the genus was divided into 2 sections, annual species in sect. *Adonis* and perennials in sect. *Consiligo* DC, and is represented by 11 taxa in Turkey [4, 12–15]. Previous studies on *Adonis* were mostly restricted to morphological [16, 17], ecological [18], palynological [19] and cytological analyses [20]. Large variations in morphological characteristics and hybridizations between some species are the main problems in identification of *Adonis* species. Many previous studies reported that molecular

techniques had great contributions to understanding phylogeny, evolution and taxonomy of *Adonis* and the other species [21–23]. Recently, Ro et al. [24] investigated phylogenetic relationships among 31 species belonging to Ranunculaceae family distributed in America using ITS regions (26S DNA) and reported that *Adonis vernalis* and *Trollius laxus* species were closely related. Johansson [21] studied phylogenetic relationships of *Adonis amurensis*, *A. annua*, *A. brevistyla*, *A. cyllenea*, *A. pyrenaica* and *A. vernalis* species with *rps16* regions and reported that these species were close relatives and total size of genome was 151.3 kb in *A. annua* and 156.5 kb in *Adonis vernalis*. Suh et al. [25] studied phylogenetic relationships among 60 genotypes and 12 populations belonging to *A. amurensis*, *Adonis pseudoamurensis*, *A. multiflora*, and *A. vernalis* in Korea based on ITS and 5.8S regions in nuclear DNA. In that study, systematic status of *A. pseudoamurensis* taxon, which was previously introduced as a new species based only on morphological data, was supported with the molecular data. Cai et al. [26] studied phylogeny of 82 species including *A. amurensis*, *A. multiflora*, *A. ramosa* and *A. shikokuensis* of Ranunculaceae family widespread in China with the aid of ITS and 5.8S regions. With that study, previously classified sub-families and tribes based on morphological data were revised according to the phylogenetic tree obtained from molecular data. Despite several previous studies, molecular studies on *Adonis* generally focused on perennial species and the information about phylogeny and genetic diversity of this genus is quite limited. The main objective of this study was to analyze genetic diversity and phylogenetic relationships among and within *Adonis* genus naturally growing in Turkey based on nrDNA ITS. To the best of our knowledge, our findings reflect the great genetic diversity of *Adonis* populations and molecular data obtained from this study can be used to explore the genetic variation pattern, population structure, and the evolutionary history of natural *Adonis* species in the future.

Materials And Methods

Plant samples

During the field studies for the taxonomic revision of the genus in Turkey, plant samples were collected from 21 natural populations of *Adonis* L. (*A. volgensis* Stev. ex DC., *A. paryadrica* (Boiss.) Kandemir & Aytaç, *A. aleppica* Boiss., *A. annua* L., *A. microcarpa* DC., *A. dentata* Del., *A. aestivalis* L. subsp. *aestivalis* L., *A. aestivalis* L. subsp. *parviflora* (Fisch. ex DC.) Busch., *A. eriocalycina* Boiss., and *A. flammea* Jacq.) in different regions between the years 2014 and 2018 (Fig. 1 and Table 1). Botanical identification of all samples was carried out based on the related literature [4, 8, 9, 13, 16, 27–35].

Table 1
Sampling locations and geographic distribution of *Adonis* populations in this study.

No	Taxa	Latitude	Longitude	Altitude (m)	Locality
1	<i>A. volgensis</i> 161	39°76'870"	44°14'774"	1580	B9 Iğdır: Elmagöl
2	<i>A. volgensis</i> 167	40°46'264"	42°95'340"	1827	A9 Kars: Kağızman
3	<i>A. paryadrica</i> 179	40°21'289"	38°57'439"	2276	A7 Giresun: Alucra
4	<i>A. paryadrica</i> K10548	36°60'230"	39°09'888"	1900	B7 Erzincan: Kemah
5	<i>A. aleppica</i> 117	37°39'885"	38°44'698"	576	C7 Urfa to Bozova road
6	<i>A. aleppica</i> 141	36°93'452"	37°38'829"	803	C6 Antep to Kilis road
7	<i>A. annua</i> 123	37°53'903"	36°82'373"	489	C6 Maraş-Süleymanlı
8	<i>A. annua</i> 143	36°93'452"	37°38'829"	803	C6 Antep-Kilis road
9	<i>A. microcarpa</i> 102	36°61'688"	36°57'067"	224	C6 Hatay: Kırkhan
10	<i>A. microcarpa</i> 122	37°53'898"	36°82'374"	493	C6 Maraş: Narlı
11	<i>A. dentata</i> 108	36°80'804"	36°93'789"	476	C6 Kilis to Hassa road
12	<i>A. dentata</i> 114	37°01'059"	38°03'994"	494	C7 Urfa: Bentbahçesi
13	<i>A. aestivalis</i> subsp. <i>aestivalis</i> 144	38°27'653"	30°16'245"	1120	B2 Kütahya to Afyon road
14	<i>A. aestivalis</i> subsp. <i>aestivalis</i> 148	38°01'864"	34°05'018"	1174	B5 Aksaray to Adana road
15	<i>A. aestivalis</i> subsp. <i>aestivalis</i> 168	39°97'982"	41°47'076"	1830	B8 Erzurum: Pasinler
16	<i>A. aestivalis</i> subsp. <i>parviflora</i> 115	37°01'059"	38°03'994"	494	C7 Urfa: Bentbahçesi
17	<i>A. aestivalis</i> subsp. <i>parviflora</i> 129	37°05'282"	38°08'719"	554	C7 Urfa: Birecik-Suruç
18	<i>A. eriocalycina</i> 132	37°37'213"	40°70'214"	980	C8 Mardin: Zinnar
19	<i>A. eriocalycina</i> 164	40°28'104"	42°95'687"	1820	A9 Kars: Kağızman
20	<i>A. flammea</i> 135	37°37'213"	40°70'214"	980	C8 Mardin: Zinnar
21	<i>A. flammea</i> 154	39°61'516"	32°65'337"	1063	B4 Ankara: Haymana

Morphological analysis

Adonis species should be collected during the growing season since the morphological characteristics such as achene and flower are very important easy to identify (Fig. 2). In our study, morphological analysis was performed based on Flora of Turkey using the following features: flower diameter, sepal shape, sepal width and length, feather in sepal, number of petals, petal shape, petal color, petal width and length, blackish in petal base, aggregate width and length, surface type of achene, achene width and length, hump position, hump shape, hump width and length, beak width and length, beak shape, beak surface and beak color.

DNA extraction, PCR amplification, sequencing

Total genomic DNA from each accession was extracted as previously described by [36]. The quality of DNA was confirmed by electrophoresis in 0.8% agarose gel, and the DNA concentration was measured using The NanoDrop® ND-1000 UV/Vis spectrophotometer. The final DNA concentration was adjusted to 50 ng/μL for ITS analysis, and the diluted DNA was stored at -20°C. PCR reactions were prepared using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), P16 (5'-CCAYTGAACCTTATCATTKAGAGGA-3') and P25 (5'-GGGTAGTCCCGCCTGACCTG-3') primers from previous reports [37–39]. PCR amplifications were performed in a thermal cycler (Labcycler). The PCR mixture consisted of 1 X buffer, 2 mM MgCl₂, 0.25 mM of each dNTP, 1 μM (20 pmol) primer, 0.5 U Taq polymerase, and 50 ng/μL DNA template in a 20 μL reaction mixture. The amplification conditions were as follows: an initial denaturation step of 3 min at 95°C, 38 cycles of 60 s at 95°C, 60 s at 67°C, 120 s at 72°C, and a final extension step of 10 min at 72°C. The amplification products were resolved in 1.5% agarose gel in 1 X SB buffer at 6 V/cm for 120 min, stained with ethidium bromide (0.5 ug/mL), and visualized under a UV-trans illuminator. The sizes of the base pairs were determined based on a DNA ladder between 50 and 1.000 bp (Vivantis Product No: NM2421). PCR products were sequenced using an ABI 3500XL (Applied Biosystems, Foster City, CA, USA) automated sequencer.

Molecular cloning and sequencing

The raw data obtained from the sequencing process were edited using ChromasPro Version 1.7.5 (Technelysium Pty. Ltd. 2003-2013). The sequence alignment was performed on ClustalW 2.1 program [40] and adjusted manually. The phylogenetic tree was constructed using the Neighbor Joining Tree-Jukes-Cantor model of Geneious V. 11.1.4 program. The phylogenetic tree was visualized using Interactive Tree of Life [41]. Branch support values were calculated using a full heuristic search using maximum number of trees 1000 and 1000 bootstrap replicates. ITS sequences of *Ranunculus asiaticus* L. (GU257963), *Delphinium polycladon* Eastw (AF258743), and *Adonis vernalis* (AJ347910) taxa used as out-group in the phylogenetic tree were retrieved from NCBI GenBank based on related studies [26, 42–44].

Results And Discussion

In this study, about 600 bp DNA regions were obtained from 64 genotypes of 10 *Adonis* taxa natural distributed in Turkey. The data for investigated *Adonis* species and out-groups were subjected to genetic distance clustering analysis and resultant dendrogram is presented in Fig. 3.

According to resultant dendrogram, outgroups are clearly different from the examined *Adonis* species. In dendrogram, *Adonis* members were separated into two large groups based on their genetic distance to each other. In the first large group, *Consiligo* section including perennial *A. volgensis* and *A. paryadrica* species was close to perennial out-group taxa *A. vernalis*, *Adonis* section composed of annual species was clearly separated from them. In the second large group, annual *Adonis* species were placed together close to each other. Such a case proved that genotypes of Turkish flora and present *Adonis* taxa were separated at section-level based on their length of life and general morphology. Considering the annual *Adonis* section, it was observed that *A. aleppica*, *A. microcarpa*, *A. dentata*, and *A. annua* species (from *A. aleppica* FK117-1 to *A. annua* FK143-2) were placed in the 1st main group of large main groups and the other species (from *A. flammea* FK135-2 to *A. aestivalis* subsp. *aestivalis* FK144-3) were placed in the 2nd main group. *A. microcarpa* generally has red flowers and rarely has yellow flowers. Red-flower samples were largely close to *A. annua* species). Yellow-flower *A. microcarpa* FK122-4 was very similar morphologically to *A. dentata*, thus was placed in the same group with it. Although yellow-flower forms of this species are weakly monophyletic, such a case should be elucidated with further studies (Fig. 3).

Genetic similarity data were given in supplementary file. There were quite high genetic similarity coefficients between yellow-flower *A. microcarpa* and *A. dentata* (98.46%) and they had very close relationships with each other. Morphological characteristics facilitating phylogenetic separation of the taxa are presented in Fig. 3. Genetic distances among *Adonis* species varied between 1.16-26.43%. Based on only primers used in this study, the lowest genetic distance ratios were respectively observed as 1.16% (between *A. microcarpa*-102 and *A. annua*-123) and 1.21% (between *A. microcarpa*-122 and *A. dentata*-108); the greatest genetic distance ratios were respectively observed as 26.43% (between *A. volgensis*-167 and *A. aestivalis* subsp. *aestivalis*-144), 26.215 (between *A. paryadrica*-179 and *A. aestivalis* subsp. *aestivalis*-144) and 22.975 (between *A. aestivalis* subsp. *parviflora*-129 and *A. volgensis*-167). With regard to intraspecific genetic distances, the lowest values were observed in *A. annua* (0.42%), *A. dentata* (0.43%) and *A. eriocalycina* (1.15%); the greatest values were observed in *A. aestivalis* subsp. *aestivalis* (19.01%) and *A. volgensis* (5.02%). The dendrogram revealed that grouping of the species of the genus largely depended on morphological structure. The *A. volgensis* and *A. paryadrica* species of *Consiligo* section were perennial and they were quite similar in rhizome structure, flower diameter and color, number of petals, aggregate and achene structures. Since *A. aestivalis* sub-species were morphologically similar, they were also in similar phylogenetic grouping. While *A. aestivalis* subsp. *aestivalis* and *A. aestivalis* subsp. *parviflora* had similar general morphology, aggregate structure, achene shapes, flower colors and petal stains, they were different in flower diameter, achene sizes and surfaces. *A. annua* and *A. microcarpa* species closely positioned in the dendrogram were similar in hairs, spread zones, flowering periods, non-hairy stems, sepal hairs, flower color, petal stains, achene sequence, size and surface, but different in achene shape, dorsal hump, flower diameter and sepal shape (Fig. 2).

In this study, intra and inter-species genetic relations of *Adonis* species were determined with the aid of molecular data gathered from 10 *Adonis* taxa widespread in Turkey, an out-group *Ranunculus asiaticus* taxa morphologically close to *Adonis* and *Delphinium polycladon* taxa morphologically far from *Adonis*. In Flora of Turkey [13], based on morphological characteristics, perennial *A. volgensis* and *A. paryadrica* species were separated from annual species in number of petals, flower colors and aggregate structures and placed under sect. *Consiligo*. The present phylogenetic dendrogram also supported such a separation. Heyn and Pazy [20] reported number of chromosomes as $2n=16$ for *A. annua* and *A. dentata*, as $2n=32$ for *A. microcarpa* and as $2n=48$ for *A. aestivalis*. According to the present findings, *A. annua* and *A. dentata* species with the same number of chromosomes were genetically quite close to each other (98.44% similarity coefficients). In the study of Cai et al. [26] and Wang et al. [42], *Adonis* species were genetically placed together with *Ranunculus* species in the same group and separated from *Delphinium* species. In the present dendrogram, *Adonis* species were placed at close positions to morphologically close *Ranunculus asiaticus* species (Fig. 3). In Kandemir et al. [15], the status of the *Adonis cyllenea* var. *paryadrica* was re-evaluated and it was decided to change its taxonomic status to species level as *A. paryadrica* according to leafy stems, young carpels more densely pubescent, sepals $\frac{1}{2}$ to petals, and tall rhizomatous. In our results, taxonomic status of *A. paryadrica* was supported with molecular data. Fischer et al. [45] indicated increasing gene flow between the populations with decreasing geographical distances. Present dendrogram also revealed high genetic similarities between geographically close populations. For instance, while the genetic similarity of Erzurum population of *A. aestivalis* subsp. *aestivalis* (FK 168) to geographically close Aksaray population (FK 148) was 90.59%, genetic similarity to further Kütahya population (FK 144) was 84.33%. Mitton and Grant [46] and Bosh et al. [47] indicated the most significant factors threatening endemic and endangered plant species as changing ecological conditions and potential genetic risks resulting from reduced genetic variation [22]. In the present study, genetic similarity between endemic *A. paryadrica* populations close species was quite high. Right at this point, it was proved once again that calculation of genetic variation played a significant role in decisions to be made for the preservation of endemic and endangered species.

Conclusions

Our molecular findings on genetic diversity of different *Adonis*'s species can be informative and used to explore the genetic variation pattern, population structure, and the evolutionary history of natural plant species in the future. Molecular data obtained by ITS primers can provide useful information to deal with various aspects of taxonomic classification of *Adonis* and the natural plant species. Along with the evolutionary process, it is known that there are some variations between species and species in plants necessitating revision studies. This paper is the first report the employing ITS primers on *Adonis* L. (Ranunculaceae) for study genetic variation of various ecological sites of Turkey. In the present study, it was determined that the taxonomic classification of *Adonis* of the ITS sequences is highly conserved among the species in this study, supporting successfully reconstructing the phylogenies at the species level. It has been concluded that the ITS sequences of nrDNA of *Adonis* provide enough data to identify and classify the economically relevant species along with the distinctive structural characters.

Declarations

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Author contributions FK and Aİ conceived and designed the study; FK and Aİ developed the plant material; FK, Eİ and AT performed the DNA extraction, analyzed the ITS data; FK and KH statistical analysis; FK, Eİ and AT drafted the manuscript; FK, Eİ and AT revised the manuscript. All authors agree and approved the final version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All samples were collected from commercial landings, and no ethical approval was required.

Consent to participate

Not applicable.

Consent for publication

All the authors agree to submit and publish an article entitled “Molecular phylogeny based on its sequences of nrDNA ITS of *Adonis* L. (Ranunculaceae) from various ecological sites of Turkey” in Molecular Biology Reports.

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Figures

Figure 1

Geographical locations of *Adonis* populations in the study.

Figure 2

Enlarged view of flowers of (a) *Adonis vernalis*, (b) *A. paryadratica*, (c) *A. aleppica*, (d) *A. annua*, (e) *A. microcarpa*, (f), *A. dentata* (g), *A. aestivalis* ssp. *aestivalis*, (h) *A. aestivalis* ssp. *parviflora* (i) *A. eriocalycina* and (j) *A. flammea*

Figure 3

Pylogenetic dendrogram of *Adonis* ecotypes based on nrITS DNA sequences and the related sequences retrieved from NCBI GenBank

Supplementary Files

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