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Phylogenetic study of *Adonis* in Iran using nuclear and plastid DNA data

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Abstract

The genus *Adonis* is predominantly distributed in the temperate regions of the Northern Hemisphere. The present study is aimed to elucidate the phylogenetic relationship within the Iranian species of the genus *Adonis*. A molecular phylogenetic approach, based on nrDNA ITS and cpDNA *trnL-F* sequences, was applied to six species, one subspecies, and three varieties. Phylogenetic analyses were conducted using parsimony and Bayesian inference methods implemented in MEGA and PAUP softwares. The results confirmed the monophyly of *Adonis* sect. *Adonis*, consistent with previous studies. In the phylogenetic tree inferred from nrDNA ITS data, species of *Trollius* clustered together with the perennial species of *Adonis* (sect. *Consiligo*) in a single clade, whereas seven taxa of *Adonis* (sect. *Adonis*) formed a separate clade. In contrast, the cpDNA *trnL-F* data revealed that, all the species of *Adonis* (sect. *Adonis* and sect. *Consiligo*) grouped together in a single clade, while the perennial species of *Trollius* formed a distinct clade. In this analysis, the two clades were separated with 97% bootstrap support. Furthermore, the taxonomic position of *A. globosa* as a subspecies of *A. microcarpa* and *A. microcarpa* as a distinct species was confirmed.

Keywords: Bayesian inference, molecular systematics, nuclear ribosomal DNA, parsimony analysis, Ranunculaceae, sequence variation

مطالعه تبارزایی جنس *Adonis* در ایران با بهره‌گیری از داده‌های DNA هسته‌ای و پلاستیدی

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خلاصه

جنس چشم‌خروس (*Adonis* L.) از آلاله‌ئیان عمدتا در نواحی معتدل نیمکره‌شمالی پراکنش دارد. هدف از پژوهش حاضر روشن‌سازی روابط تبارزایی درون‌جنس *Adonis* در ایران بود. به این منظور، از رویکرد تبارزایی مولکولی بر پایه توالی‌های nrDNA ITS و cpDNA *trnL-F* در شش گونه، یک زیرگونه و سه واریته استفاده شد. تحلیل‌های تبارزایی با بهره‌گیری از روش‌های پارسیمونی و آنالیز بیزین در نرم‌افزارهای MEGA و PAUP انجام گرفت. نتایج تک‌تبار بودن بخش *Adonis* تأیید کرد که با مطالعات پیشین نیز هم‌خوانی دارد. در درخت تبارزایی حاصل از داده‌های nrDNA ITS، گونه‌های *Trollius* به همراه گونه‌های چندساله *Adonis* (بخش *Consiligo* DC.) در یک کلاد قرار گرفتند، در حالی که هفت آرایه از *Adonis* (بخش *Adonis*) کلاد مجزایی را تشکیل دادند. در مقابل، داده‌های cpDNA *trnL-F* نشان داد که همه گونه‌های *Adonis* (هر دو بخش *Adonis* و *Consiligo*) در یک کلاد جای گرفتند، به طوری که گونه‌های چندساله *Trollius* کلاد مستقل و متمایزی را تشکیل دادند. در این تحلیل، جدایی دو کلاد با بوت‌استرپ ۹۷ درصد پشتیبانی شد. افزون بر این، جایگاه رده‌بندی *A. globosa* C. Steinb. ex Rech.f. به عنوان یک زیرگونه و *A. microcarpa* DC. به عنوان یک گونه مستقل مورد تأیید قرار گرفت.

واژه‌های کلیدی: تحلیل بیزین، تحلیل پارسیمونی، تنوع توالی، دی.ان.آ. ریبوروم هسته‌ای، سیستماتیک مولکولی، Ranunculaceae

Introduction

The genus *Adonis* L. (Ranunculaceae) comprises 35 species worldwide (Tamura 1991, Wang 1994), with seven species occurring in Iran (Pakravan & Sharifnia 2023). Its distribution is primarily concentrated in the temperate regions of the Northern Hemisphere, though a few annual species extend into N. Africa.

This genus has pinnately dissected, alternate leaves with actinomorphic, yellow or red solitary flowers and cylindrical or globular aggregated achenes. Taxonomically, *Adonis* is readily divided into two groups: annual and perennial species. De Candolle (1818) originally classified annuals within the sect. *Adonis* and perennials in the sect. *Consiligo*. However, some botanists have proposed segregating perennial species into separate genera (*Adonanthe* Spach and *Chrysocyanthus* Falk.). Based on morphological characteristics, Wang (1994) retained all species within *Adonis* but recognized two subgenera: *Adonis* (annual species) and *Adonanthe* (Spach) W.T. Wang (perennial species).

In addition to morphological differences, annual and perennial species exhibit distinct chromosomal structures and pollen grain types. Imam *et al.* (1977) proposed that, annual species tend to be polyploid, whereas perennial species are typically diploid. Furthermore, studies by Ghorbani *et al.* (2008) and Gostin (2009) have identified distinct pollen grain types associated with annual and perennial species, further differentiating the two life-history strategies.

Annual species present significant taxonomic challenges (Steinberg 1971) due to the high similarity in vegetative organs, petal shape, and color-traits that can further change in herbarium specimens (Riedle 1963). Even fruit characteristics, a key diagnostic feature for many taxonomists, vary depending on ripening stage and regional climatic conditions (Riedle 1963). Compounding these difficulties, hybridization between species has led to populations with intermediate traits, prompting botanists to describe numerous subspecies and varieties. As a result, species identification becomes particularly problematic when relying solely on herbarium specimens without field observations of natural populations. This has contributed to inconsistencies in species concepts across different floras.

Recent advances in molecular research have significantly contributed to resolving species' taxonomic positions and phylogenetic relationships (Baldwin 1992). In addition, previous molecular studies on other Iranian plant families also confirmed the value of molecular phylogeny in resolving taxonomic relationships (Escobar Garcia *et al*. 2012, Fereidounfar *et al*. 2016). However, studies on *Adonis* perennial species have produced conflicting results, as evidenced by the works of Wang (1994) and Son *et al*. (2016). Meanwhile, Najariyan *et al.* (2020) investigated annual *Adonis* species using ITS sequencing and ISSR markers, revealing a monophyletic origin for annuals, though their outgroup (*Trollius*) was unexpectedly nested within perennial lineages.

Despite these efforts, taxonomic ambiguities persist among *Adonis* species in Iran, with prior phylogenetic analyses failing to resolve the status of certain subspecies. To address these uncertainties, the present study aims to clarify the systematic position and phylogenetic relationships of Iranian *Adonis* species through a combined approach, analyzing nrDNA ITS and cpDNA *trnL-F* sequence variations alongside morphological data.

Materials and Methods

- Taxon sampling

For the molecular phylogenetic analysis, seven taxa of the genus *Adonis*, including six species, one subspecies and three varieties following the results of Hoot (1995) were sampled. Samples were obtained from field collections and herbarium specimens. Vouchers of specimens were deposited at the herbaria of Alzahra University (ALUH), Kharazmi University (FARABI), and Tehran University (TUH), Tehran, Iran (Table 1).

The identification of the specimens was carried out based on the Flora of Iran (Pakravan & Sharifnia 2023). Nucleotide sequences for the nuclear ribosomal ITS region and the chloroplast *trnL-F* intergenic spacer

were acquired for species of *Trollius* and *Adonis* (sect. *Adonanthe*). *Thalictrum majus*, *Consolida orientalis*, and *Caltha palustris* were selected as outgroups. All sequences were downloaded from the National Center for Biotechnology Information (NCBI) database, GenBank.

- DNA extraction, PCR, sequencing, and sequence alignment

Total genomic DNA was extracted either from silica-gel-dried leaves of specimens collected in the wild or from herbarium specimens following the manufacturer's protocols of the Plant Total DNA Extraction Kit (Bioer Co., China). Polymerase chain reaction (PCR) amplifications of the whole region of nr DNA ITS and cp DNA *trnL-F* were performed using the following primers: ITS4 and ITS5 (White *et al*. 1990), and *trnL* and *trnF* (Taberlet *et al.* 1991). PCR amplification was conducted according to the protocol described by Cai *et al.* (2009) using a Mastercycler gradient thermal cycler (Eppendorf Co., Hamburg, Germany). PCR products were checked on 1% D-1, low EEO agarose gel (Pronadisa), and stained with ethidium bromide. Cycle-sequencing reactions were performed under conditions of BigDye terminator cycling (ABI sequencer 3730, ABI Co., USA), following the manufacturer's manual. The new nr DNA ITS sequences were submitted to the EMBL nucleotide sequence data base. Sequence alignment was implemented in BioEdit Ver. 7.05.2 (Hall 1999), and the alignment was optimized manually. The gaps were treated as missing data.

In the next step, these data were formatted appropriately in fasta format for input into the software MEGA 5 (Nei & Kumar 2000, Tamura *et al*. 2001) and PAUP Ver. 4.0b10 (Swofford 2001) using EditPad Lite Ver. 6.3.1.0. Subsequently, phylogenetic trees were constructed using the Maximum Parsimony (MP) method through heuristic search, which included random sequence additions. It should be noted that, in the analysis of nrDNA ITS, sequence data for 13 taxa, and in the analysis of the cpDNA *trnL-trnF* intergenic region, sequence data for seven taxa were obtained from the GenBank database. The accession numbers for these sequences are provided in table 2.

- Phylogenetic analysis

Maximum parsimony (MP) analysis of the nrDNA ITS (ITS4 +ITS5), cpDNA (*trnL* + *trnF*), was conducted using the heuristic search algorithm of MEGA Ver. 5 and combined datasets was analyzed using a Bayesian approach as implemented in MrBayes Ver. 3.1 (Huelsenbeck & Ronquist 2001).

Table 1. The list of studied Iranian taxa of *Adonis* along with related data

No.	Taxon	Locality, collector & voucher number	Herbarium name*
1	*Adonis dentata* Delile ssp. *persica* (Boiss.) H. Riedle	Fars Prov.: 20 km to Darab, Hassanpoor 11824	FARABI
2	*A. microcarpa* DC.	Fars Prov.: Kazeroon, Parishan Lake, 820 m, Rasti 11799	FARABI
3	*A. flammea* Jacq	Alborz Prov.: Taleqan, 2000 m, Shojaeinia 6360	ALUH
4	*A. aestivalis* L. var. *aestivalis*	E. Azerbaijan Prov.: Arasbaran, Veinagh, Pakravan 6362	ALUH
5	*A. aestivalis* L. var. *provincialis* (DC.) W.T.Wang	Ghazvin Prov.: 10 km to Ghazvin in from Tehran, 1300 m, Shojaeinia 6365	ALUH
6	*A. globosa* C. Steinb. ex Rech. f.	Tehran Prov.: Lavasan, 1700 m, Pakravan 4703	ALUH
7	*A. wolgensis* Stev. ex DC.	E. Azerbaijan Prov.: Arasbaran, Makeidy, Ghahreman 35418	35418 TUH

∗ ALUH: Alzahra University, FARABI: Kharazmi University, and TUH: Tehran University (TUH) herbaria (Tehran, Iran)

Table 2. The list of Accession number from GeneBank

Taxon	ITS	*trnL-F*
Adonis amurensis Regel & Radde	AF454928.1	-
A. amurensis Regel & Radde	-	FJ626534.1
A. annua L.	AY148280.1	-
A. annua L.	-	AH012590.1
A. multiflora Nishikawa & Koji Ito	AF454926.1	-
A. pseudoamurensis W.T.Wang	AF454935.1	-
A. ramose Franch.	AB361616.1	-
A. shikokuensis Nishikawa & Koji Ito	AB361623.1	-
A. vernalis L.	AJ347910.1	-
Trollius vaginatus Hand.-Mazz.	HQ440205.1	-
T. vaginatus Hand.-Mazz.	-	HQ440195.1
T. farreri Stapf	HQ440201.1	-
T. farreri Stapf	-	HQ440191.1
T. ranunculoides Hemsl.	HQ440203.1	-
T. ranunculoides Hemsl.	-	HQ440193.1
T. dschungaricus Regel.	HQ440199.1	-
T. dschungaricus Regel.	-	HQ440189.1
Caltha palustris L. var. *membranacea* Turcz.	AY515398.1	-
C. palustris L. var. *membranacea* Turcz.	-	FJ626540.1
Consolida orientalis (Gay) Schrod.	JF331896.1	-
Thalictrum majus L.	JF742162. 1	GQ245606.1

Results

- Molecular data

The aligned data matrices for the ITS and *trnL-F* regions across the seven taxa comprised 660 and 1,045 base pairs (bp), respectively. Maximum parsimony (MP) analysis of the cpDNA dataset (1045 total characters) revealed that, 650 sites were conserved, 263 were variable but parsimony-uninformative and 107 were parsimony-informative. This analysis yielded 14 most parsimonious trees with a length of 408 steps. The tree statistics were as follows: consistency index (CI) = 0.707, retention index (RI) = 0.817, and rescaled consistency index (RC) = 0.669.

The ITS dataset yielded partially unresolved trees (Fig. 1) but revealed three well-supported *Adonis* clades (BS = 98%): 1) Annual species clade containing two strongly supported subgroups (*A. dentata* ssp. *persica* + *A. microcarpa* + *A. annua* [BS = 98%], and *A. flammea* + *A. globosa* + *A. aestivalis* ssp. *provincialis* + *A. aestivalis* ssp. *aestivalis* [BS = 96%]); and 2) A perennial clade *Adonis* [BS = 99%]; and 3) *Trollius* perennials [BS = 100%], with Bayesian and MP analyses showing topological congruence. In contrast, *trnL-F* analysis (TL = 161, CI = 0.863, RI = 0.940) united annual/perennial *Adonis* in Clade 1 and *Trollius* perennials in Clade 2 (BS = 98% separation), with outgroups forming distinct clades and *T. majus* as the closest *Adonis* relative.

Parsimony analysis of the combined dataset yielded 10 most parsimonious trees (tree length = 559) with the following indices: consistency index (CI) = 0.747, retention index (RI) = 0.838, and rescaled consistency index (RC) = 0.721 (Fig. 3). The resulting phylogeny strongly separated *Adonis* and *Trollius* into distinct clades (100% bootstrap support), while maintaining the same topological relationships observed in the nrDNA analysis with

A. flammea grouped within the clade containing *A. globosa*, *A. aestivalis* ssp. *provincialis*, and *A. aestivalis* ssp. *aestivalis*.

Molecular analyses revealed that, *A. microcarpa* and *A. dentata* ssp. *persica* differ by four nucleotides in the nuclear genome and nine nucleotides in the chloroplast genome. In addition, *A. aestivalis* and *A. globosa*, also differ in their nuclear gene sequences in the ITS region (two nucleotides) as well as in their chloroplast genome sequences in the *trnL-F* region (11 nucleotides). The nucleotide composition of both ITS (nuclear genome) and *trnL-F* (chloroplast genome) regions is detailed in tables 3–4, which present the percentages and ratios of purine and pyrimidine bases for each genomic compartment.

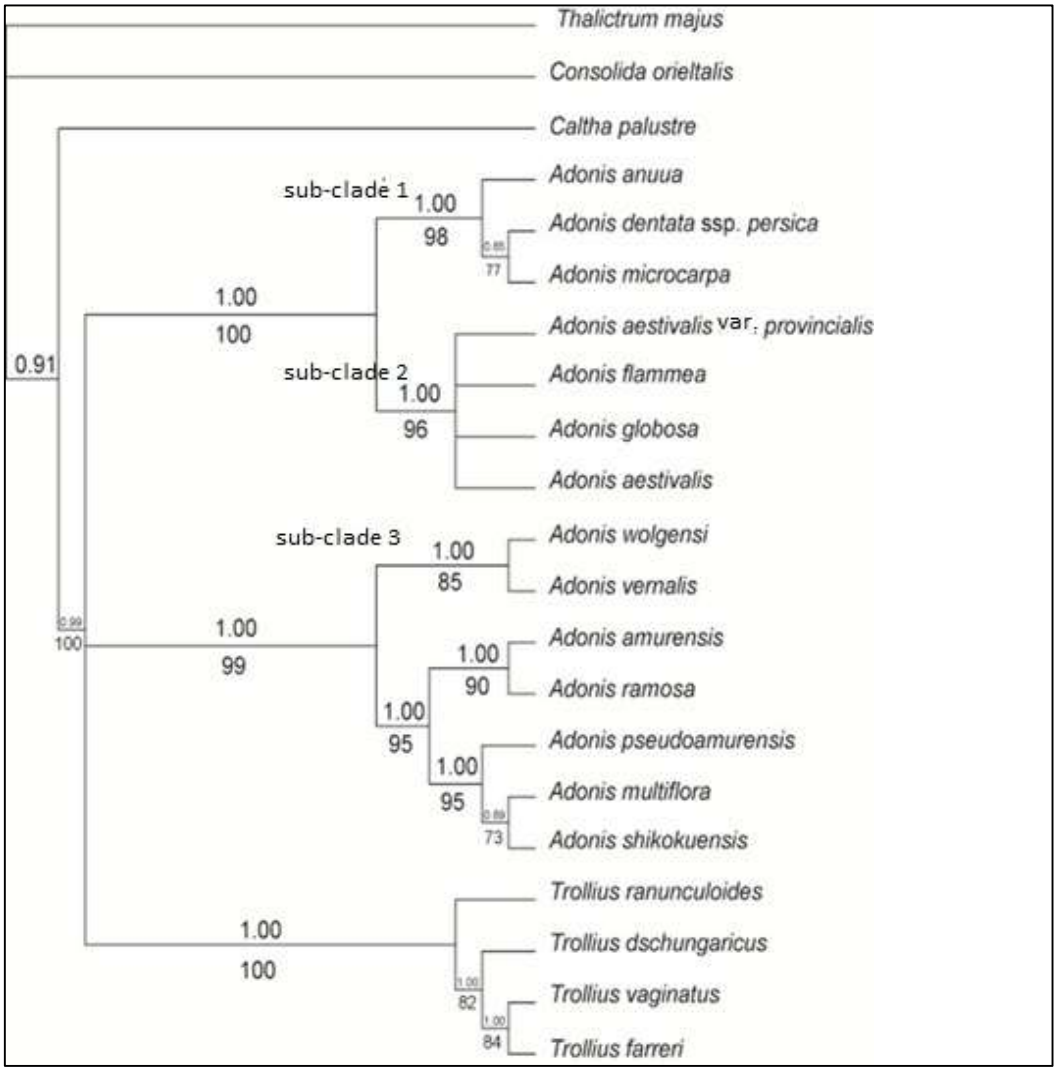

Fig. 1. The Bayesian tree resulting from the analysis of the sequenced nrDNA ITS region of the studied *Adonis* species using the MrBayes software (Clade 1: Annual species, Clade 2: Perennial species, Clade 3: *Trollius* species). Values above branches indicate posterior probability; values below branches are bootstrap support of ≥ 0.90/≥ 50.

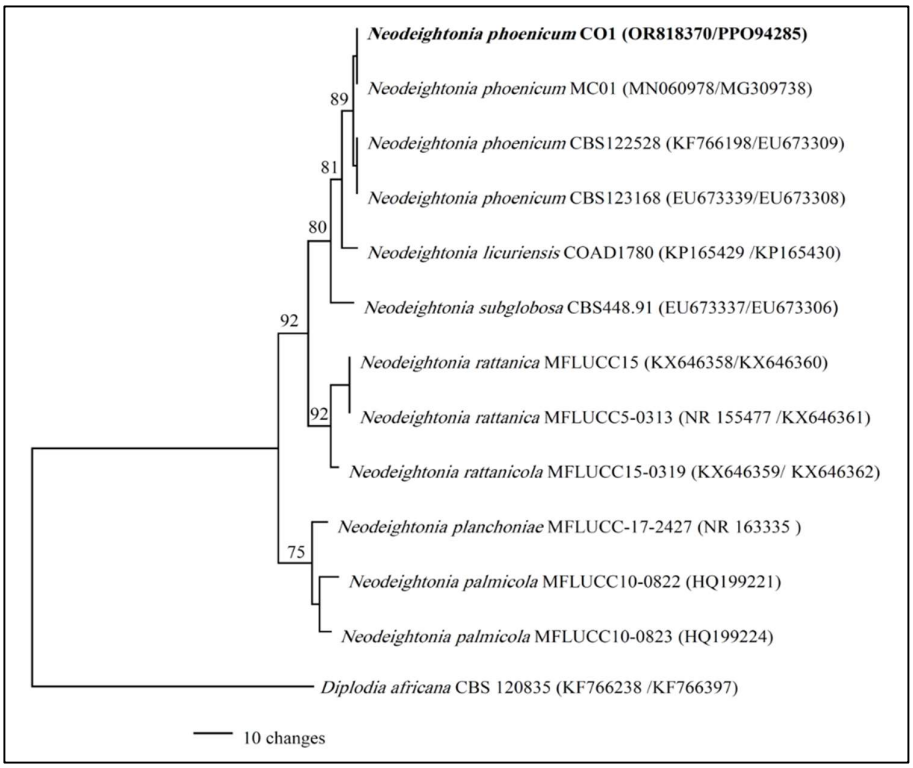

Fig 2. The Maximum Parsimony tree resulting from the analysis of the sequenced cpDNA *trnL-F* region of the studied *Adonis* species using the MEGA software (Clade 1: Annual species, Clade 2: Perennial species). Values above branches indicate bootstrap support of ≥ 0.90/≥ 50.

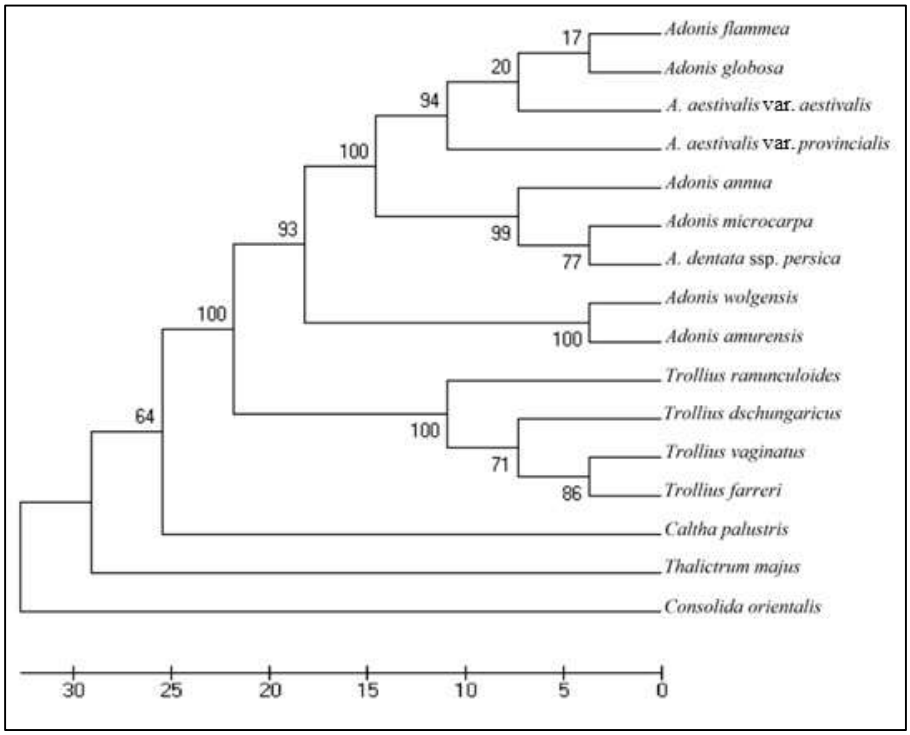

Fig. 3. The Maximum Parsimony tree resulting from the analysis of combined plastid and nuclear sequences obtained from sequencing the studied species of *Adonis* using the MEGA software (Clade 1: Annual species, Clade 2: Perennial species, Clade 3: *Trollius* species). Values above branches indicate bootstrap support of ≥ 0.90/≥ 50.

Table 3. The nucleotide composition and corresponding percentages in the nrDNA sequences of the studied *Adonis* species

Taxon	T	C	A	G	Total	A+G	T+C	A+G/T+C
A. aestivalis ssp. *provincialis*	21.8	26.6	23.8	27.8	597.0	51.59	48.41	1.066
A. aestivalis ssp. *aestivalis*	21.9	26.0	23.8	28.3	593.0	52.11	47.89	1.088
A. globosa	21.8	26.6	23.8	27.8	597.0	51.59	48.41	1.066
A. dentata ssp. *persica*	21.9	26.4	24.1	27.6	594.0	51.68	48.32	1.070
A. microcarpa	22.5	26.4	23.9	27.2	599.0	51.09	48.91	1.044
A. flammea	22.2	26.8	23.6	27.4	598.0	51.00	49.00	1.041
A. wolgensis	22.0	26.3	24.3	27.3	600.0	51.67	48.33	1.069

Table 4. The nucleotide composition and corresponding percentages in the cpDNA sequences of the studied *Adonis* species

Taxon	T	C	A	G	Total	A+G	T+C	A+G/T+C
A. aestivalis ssp. *provincialis*	30.3	17.7	37.5	14.5	821.0	52.01	47.99	1.084
A. aestivalis ssp. *aestivalis*	31.5	17.9	35.9	14.8	839.0	50.66	49.34	1.027
A. globosa	31.6	17.8	36.0	14.6	828.0	50.60	49.40	1.024
A. dentata ssp. *persica*	31.7	17.7	36.1	14.6	837.0	50.66	49.34	1.027
A. microcarpa	32.1	17.3	35.1	15.5	838.0	50.60	49.40	1.024
A. flammea	31.5	18.0	35.9	14.7	839.0	50.54	49.46	1.022
A. wolgensis	31.1	17.8	36.3	14.8	832.0	51.08	48.92	1.044

Discussion

The genus *Adonis* presents significant taxonomic challenges that are widely recognized by researchers (Riedle 1963, Iranshahr *et al.* 1992, Pakravan & Sharifnia 2023). These difficulties primarily stem from two key factors: 1) A paucity of reliable diagnostic characters; and 2) Considerable phenotypic plasticity. The primary identification challenge lies in the limited number of useful morphological characters typically only four or five prove diagnostically valuable. Compounding this issue, vegetative characters exhibit considerable variation across different habitats, floral coloration alters during desiccation and fruit characteristics are only discernible in fully mature specimens.

Floral morphology and achene surface ornamentation support taxonomic relationships that align with the two primary subclades identified in our molecular phylogenies. These findings corroborate earlier morphology-based classifications of *Adonis* (Davis 1965, Iranshahr *et al.* 1992; Fig. 2). Molecular analyses resolved three well-supported clades (Figs 2–3), each comprising species with shared morphological traits.

The chloroplast DNA (cpDNA) maximum parsimony (MP) tree (Fig. 2) supports the placement of *Trollius* as an outgroup to *Adonis*, consistent with Son *et al*. (2016). In contrast, the ITS phylogeny agrees with Najariyan *et al*. (2020), showing *Trollius* nested near perennial *Adonis* species-a result that underscores the paraphyletic nature of the perennial lineage.

Through combined analysis of cpDNA *trnL-F* and nrDNA ITS markers, three morphologically defined sub clades in *Adonis* (Fig. 1) were resolved here. Sub-clade 1 includes three species (*A. annua*, *A. dentata* ssp. *persica*, and *A. microcarpa*) characterized by small, crownless achenes, while sub-clade 2 contains four species (*A. aestivalis* ssp. *provincialis*, *A. flammea*, *A. globosa*, and *A. aestivalis* ssp. *aestivalis*) with large, prominently crowned achenes-though *A. flammea* displays a reduced crown morphology. Notably, the shared tetraploidy between *A. flammea* and *A. aestivalis* (Ghafari 1987, Reynaud 1993) supports their close phylogenetic affinity.

The consistently cluster of the diploid species *A. dentata* and *A. annua* (Gregory 1941) together with *A. wolgensis* (the sole Iranian perennial *Adonis*), forms a distinct sub-clade with other perennial species, confirmed its taxonomic differentiation from annual relatives.

Adonis aestivalis is a highly polymorphic species, for which numerous subspecies and varieties have been described by various botanists. This species is characterized by large orange to red flowers, large fruits with a prominent crown, a long beak, and a fruit surface that ranges from rugose to honeycombed (alveolate) (Fig. 4). Different varieties such as var. *parviflora*, var. *provincialis*, and var. *scrubicolata* have been defined based on the degree of dorsal protuberance, surface ornamentation of the fruit, and the shape of the beak. On the basis of the diagnostic traits described above, some specimens can be tentatively assigned to the aforementioned varieties However, in many cases, no consistent correlation among the traits was observed, making it impossible to reliably place these specimens within a specific variety. For instance, the wing-like keel, which has been described as a diagnostic feature of *Adonis* var. *scrobiculata* in the distal region of the fruit, was consistently observed across all examined specimens, albeit with considerable variation in size. This finding suggests the presence of morphological diversity in fruit shape. Considering that pollination in *Adonis* species is insect-mediated, the observed variability may reasonably be attributed to natural hybridization.

Molecular evidence supports the taxonomic distinction between *A. aestivalis* and *A. globosa*, with two nucleotide differences in the ITS region (nuclear genome) and eleven in the *trnL-F* region (chloroplast genome), potentially supporting *A. globosa* as a subspecies of *A. aestivalis*. Furthermore, between the two subspecies *A. aestivalis* ssp. *aestivalis* and ssp. *provincialis*, there are also differences of two nucleotides in the nuclear genome and six nucleotides in the chloroplast genome.

There are differing opinions regarding the taxonomic status of *A. microcarpa*. De' Candolle described this taxon from Europe and distinguished it from *A. dentata* based on several morphological characteristics, including ovate sepals (linear in *A. dentata*), reddish petals (yellow in *A. dentata*), larger fruits with a shorter dorsal protuberance and a stylar canal separate from the carpels (adnate to the carpels in *A. dentata*), and the presence of a membranous wing on the fruit (Iranshahr *et al.* 1992). Riedle treated this taxon as a subspecies of *A. dentata*. However, other botanists consider it a distinct species (Davis 1965, Iranshahr 1992). Since petal color fades to yellow upon drying in herbarium specimens, reliable identification requires field observation. Molecular analyses reveal four nuclear and nine chloroplast genome differences between these taxa, supporting the recognition of *A. microcarpa* as a distinct species. The nucleotide composition and corresponding percentages in the nrDNA and the cpDNA sequences of the studied *Adonis* species are presented in tables 3–4.

The phylogenetic analyses (ITS, MP consensus, and combined trees) in the present study consistently support the monophyly of *Adonis*, corroborating the findings of Najariyan *et al*. (2020). However, to further refine the phylogenetic reconstruction and clarify interspecific relationships within the genus, future studies should incorporate additional specimens and employ multiple molecular markers.

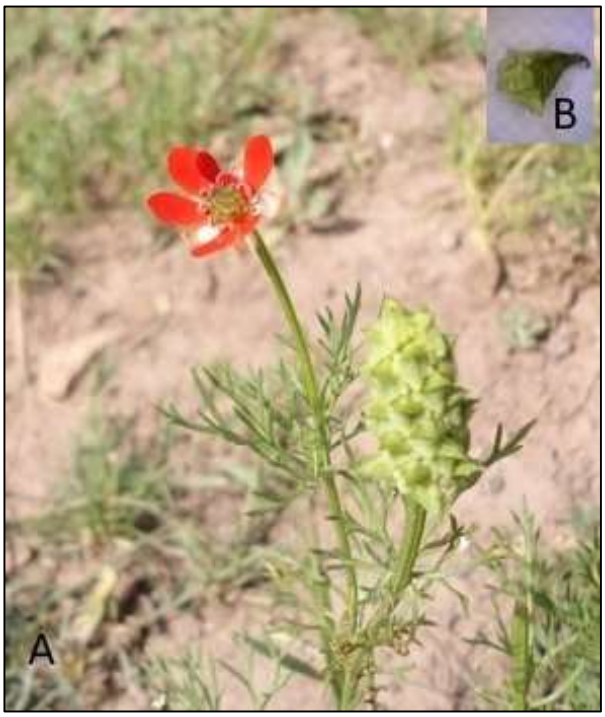

Fig. 4. *Adonis aestivalis*: A. Habitat , B. Achene (Photo by E. Shojaeinia).

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