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Original scientific article

MOLECULAR PHYLOGENY OF THE SECT. *ADONIS* (Genus *Adonis* L., Ranunculaceae)

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The ornamentally important genus *Adonis* L. (Ranunculaceae) consists of about 30 species. In Iran, 8 species are recognized. The new classification system recognizes two subgenera, six sections, and six series for *Adonis*. The species differentiation within the genus is controversial due to morphological overlaps in different species and extensive morphological variation. Therefore, the aims of present study were: 1- Species delimitation within the Sect. *Adonis* based on morphological and molecular evidences and, 2- Studying the species relationship based on molecular phylogeny. Multivariate analyses of morphological and molecular (ITS and ISSR) data differentiated the *Adonis* species. PCA biplot analysis of morphological characters revealed morphological characters such as leaf arrangement; leaf length, flower diameter, petal length and width, calyx length, and pedicel length are of taxonomic implication in *Adonis*. Bayesian tree of ITS sequences revealed that the sect. *Adonis* is differentiated from the sect. *Adonanthe*. Based on ITS data, *A. microcarpa* showed close affinity to *A. scrobiculata*, while *A. aestivalis*, *A. icrocarpa* and *A.dentata* were close to each other.

Key words: Adonis; ITS; ISSR; Molecular phylogeny.

INTRODUCTION

The ornamentally important genus *Adonis* Linnaeus (Ranunculaceae) consists of about 30 annual or perennial species (SON *et al.*, 2016). The distribution of the genus mainly extends from Europe to far eastern Asia including Himalayas (WANG, 1994a), but some annual species are rarely found in southwestern Asia, northern Africa, and Mediterranean region (TAMURA,

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1991). Considering the geographical distribution of the genus as well as that of its most primitive species *A. nepalensis* Simonov., it may be deduced that there are three geographic routes of speciation that it has taken: from the western Himalayas to Europe, from the southwestern China to eastern Siberia via northeastern China, and from the southwestern China to northern Asia (WANG ,1994b; SON, 2015).

The genus is characterized by palmately or 2–3 pinnately dissected alternate leaves and the stem base to a membranous sheath or scale. Terminal flowers are yellow or red with 3–30 petals and have numerous stamens and many pistils. The aggregated fruiting heads are globose to cylindrical, and achenes retain a short style after ripening (CHANG *et al.*, 2008).

A few species in the genus *Adonis* are the only land plants known to produce the valuable red ketocarotenoid astaxanthin in abundance. The pathway that leads from the β -rings of b-carotene, a carotenoid ubiquitous in plants, to the 3-hydroxy-4-ketob- rings of astaxanthin (3,39-dihydroxy-b, b-carotene-4,4'-dione) was reported in the blood-red flowers of *Adonis aestivalis* L., an ornamental and medicinal plant commonly known as summer pheasant's eye (CUNNINGHAM, 2011). The genus *Adonis* contains 8 species in Iran (RECHINGER, 1992).

WANG (1994a) conducted a morphological study of all *Adonis* taxa and proposed a new classification system, recognizing one genus, two subgenera, six sections, and six series. He divided the genus into two subgenera, *Adonanthe* and *Adonis*. He assigned subgenera *Adonanthe* and *Adonis* to perennial and annual groups, respectively. The former has been claimed to be more primitive than the latter. The species within these subgenera were characterized by the pubescence of achene, leaflet shape, the length of style, and the presence/absence of petiole.

Different approaches have been used to investigate species differentiation and phylogeny in *Adonis*. For example, SON *et al.* (2017) conducted parsimony analysis based on morphological characters in 11 East Asian taxa (sect. *Adonanthe*) to study the species relationship and taxonomy of the genus. The results did not support the classification within sect. *Adonanthe* proposed by WANG (1994a). This is because the taxonomic criteria were based on the shape and type of leaves as well as petal color while, in the process, overlooking the fact that sect. *Adonanthe* plants living in different ecological environments possess various morphological characters and undergo different evolutionary trends, demonstrating the limitations of the current taxonomy for the genus *Adonis*.

GHORBANI *et al.* (2012) used anatomical features for species delimitation in *Adonis flammea* Jacqui, *A. aestivalis* L. and *A. dentata* Delile. Cluster analysis of several populations in these three anuual species could not clearly delimit the studied taxa. However, population anatomical variability within each species was considered to indicate sub-specific taxonomic ranks with the studied species.

CHANG *et al.* (2008) used the leaf extracted flavonoids to investigate the *Adonis amurensis* complex. This complex has been a persistent source of taxonomic confusion due to the exclusive use of continuous variation in flower morphology for species definition and recognition. Nineteen flavonoids, including c-glycosylflavones, oglycosylflavones, and flavonol O-glycosides were characterized. The flavonoids isolated from flowers and leaves of the *A. amurensis* complex were not the same. This study demonstrated that the flavonoid chemistry may indeed be as variable as the morphological features, and no significant flavonoid differences at the species level were observed.

SUH *et al.* (2002) used a combination of random amplified polymorphic DNA (RAPD) analysis and sequence analysis of internal transcribed spacer (ITS) of nuclear ribosomal DNA, to examine genetic differentiation and species delimitation of Korean *Adonis* species, while KANEKO *et al.* (2008) used *trn*L-F and the ITS region sequences to study the Japanese endemic *Adonis* plant.

SON *et al.* (2016) used DNA sequences of nrITS regions obtained from 49 accessions representing 12 species and one variety within the section *Adonanthe* of the genus *Adonis* to test the previous intra-sectional classification system and to determine their phylogenetic relationships. As indicated by SON *et al.* (2017), investigations on morphological characters and molecular and biological research on a wide range of taxa belonging to the genus is necessary to establish a broader system of natural classification not just for the East Asian sect. *Adonanthe*. Most of the previous studies regarding the genus *Adonis* L. focused on a classification system based on morphological differences, including those associated with the leaf shape and achene characters, whereas a phylogenetic relationship-based classification system of the genus *Adonis* L. has not yet been established. Furthermore, SON *et al.* (2016) showed that nrITS data did not support the classification system proposed by WANG (1994a). He classified sect. *Adonanthe* into four series, most of which were found to be either polyphyletic or paraphyletic.

In few cases the species delimitation is still in debate within the genus *Adonis*. For example, the species delimitation of the *A. amurensis* complex has been discussed by several authors (MILLAR, 1980; NISHIKAWA, 1989; SUDA and HERAI, 1991), but species delimitation is still debatable. A major difficulty was the exclusive use of continuous variation in flower morphology for species definition and recognition. Similarly, we encountered cases with synonymy, for instance *Adonis dentate* has been considered synonym to *Adonis aestivalis*. There are species with overlapping morphological characteristics which render their differentiation very difficult, for example *Adonis aestivalis* and *Adonis microcarpa* are difficult to be differentiated. Therefore, the aims of present study were: 1- Species delimitation within the Sect. *Adonis* based on morphological and molecular features, 2- Studying the species relationship based on molecular phylogeny and, 3- Investigating the monophyly of the Sect. *Adonis*.

Molecular data may provide useful and additional support for species delimitation where morphological characters are cryptic or overlapping among the species complexes (MILLAR, 2011). A combined approach should enable more reliable taxonomic judgments (BYRNE, 2003). Moreover, molecular data have been frequently used to establish a phylogenetic classification system. In particular, the ITS regions, which are nuclear DNA regions characterized by patterns of parental inheritance, evolve more rapidly than the coding regions, leading to higher levels of variation among closely related individuals. Thus, the ITS regions have been utilized to investigate inter-species and inter-genus relationships, as well as evolutionary trends and patterns in genetic variation (BALDWIN, 1991; ÁLVAREZ and WENDEL, 2003).

MATERIALS AND METHODS

Plant materials

Plant specimens of 7 *Adonis* species (*Adonis* section) were used for morphological and ISSR as well as ITS studies. The species studied are presented in Table 1. We used *Trollius* sp. as out-group species in molecular phylogeny analyses of both *Adonis* sections. ITS sequences of

the out-group taxa and *Adonis* species (sect. *Adonanthe*) were obtained from NCBI (National Center for Biological Information). The accession numbers of taxa used have been provided (Table2).

Table1. Adonis species in for morphological and ISSR as well as ITS studies.

No	Species		No	Species	
1	*Adonis dentata*	HSBU-2018625	5	*Adonis flemmea*	HSBU-2018629
2	*Adonis aestivalis*	HSBU-2018626	6	*Adonis annua*	HSBU-2018630
3	*Adonis globosa*	HSBU-2018627	7	*Adonis microcarpa*	HSBU-2018631
4	*Adonis scrobiculata*	HSBU-2018628			

*Table2. The accession numbers of taxa in phylogeny studies***.**

No	Species	accession number
1	*Adonis coerulea*	KU570402.1
2	*Adonis vernalis*	AF454936.1
3	*Adonis sibrica*	KU570405.1
4	*Adonis bobroviana*	KU570391.1
5	*Adonis multiflora*	KU570397.1
6	*Adonis shikokuensis*	AB361609.1
7	*Trollius* sp.	AH006943

Morphological study

For species differentiation we used 11 quantitative and qualitative morphological characters (Table 3). For multivariate analyses of morphological characters, data were first standardized (mean = 1, variance = 0) (PODANI, 2000). We used maximum parsimony and PCA (Principal components analysis) for morphological analyses. PAST version 2.17 (HAMMER *et al.*, 2012) and PAUP software ver. 4 (SWOFFORD, 2002) with 1000 times bootstrapping were used.

Table 3. Morphological data of Adonis species

No	Characters	No	Characters
1	Length of petal (mm)	7	Length of internod (mm)
2	Width of petal (mm)	8	Length of leaf (mm)
3	Length of sepal (mm)	9	Width of leaf (mm)
4	Width of sepal (mm)	10	Flower diameter (mm)
5	Length of pedicel (mm)	11	Stem branched
6	Leaf arrangement		

Molecular study

Fresh leaves were collected randomly from 5-10 plants in each of the studied species. They were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (KRIŽMANM, 2006). The quality of extracted DNA was examined by running on 0.8% agarose gel.

PCR reactions were performed in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of Taq DNA polymerase (Bioron, Germany).

For ITS sequencing the following primers were used (Table 4). The amplification' reactions for ITS were performed in Techne thermocycler (Germany) with the following program: 4Min initial denaturation step (94°C), followed by 35 cycles of 1 min at 94°C; 1 min at 54°C and 2 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C.

Ten ISSR primers; (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC810, (CA) 7AT, (GA) 9C, UBC807, UBC811, (GA) 9A and (GT) 7CA commercialized by UBC (the University of British Columbia) were used. The amplifications' reactions for ISSR were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step (94°C), 30 Sec at 94°C; 1 min at 57°C and 1min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. The amplification products were visualized by running on 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Table 4. Primers for ITS sequences

Number	Primer	
1	ITS5	5′- GGA AGT AAA AGTCGT AAC AAG G- 3′
2	ITS4	5′- TCC GCT TATTGA TAT GC- 3′

The ITS sequences were aligned by MUSCLE program and used for ML (Maximun parsimony) tree construction after 100 times bootstrapping (KOOHDAR and SHEIDAI, 2019). For these analyses MEGA 7 (TAMURA, 2011) program was used.

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). AMOVA (Analysis of molecular variance) with 1000 permutations as implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006), was used to reveal significant genetic difference among the studied species. We used MDS for ISSR analyses. PAST version 2.17 (HAMMER *et al.*, 2012) was used.

RESULTS

Species delimitation and their relationship

Morphometric and molecular studies (ISSR and ITS sequences) of the studied species within sect. *Adonis* revealed the species differentiation. PCoA plot (no figure) of the studied samples based on morphological features separated *Adonis* species from each other. PCA biplot (Fig. 1) of morphological characters revealed that the first two components comprised about 92% of total variation, and characters like leaf arrangement, leaf length, flower diameter, petal length and width, calyx length, and pedicel length had the highest correlation with these axes and are the most variable morphological characters among *Adonis* species. Therefore, these characters have taxonomic value in the genus *Adonis*.

The species relationship based on morphological characters was revealed by maximum parsimony tree (Fig. 2). *Adonis flemmea* is placed far from the other studied species within the

sect. *Adonis*. This is due its whorl-type leaf arrangement, while the rest of taxa have alternate-type of leaf arrangement (Table5).

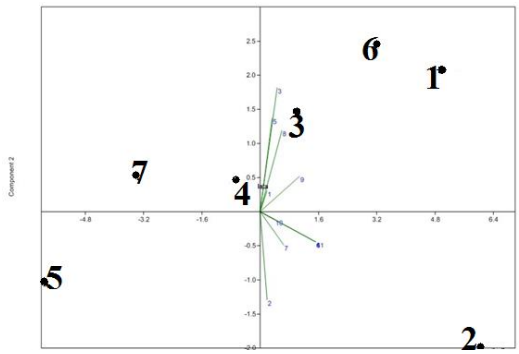

Fig.1. PCA biplot of *Adonis* species based on morphological features. (Species numbers are according to Table 1).

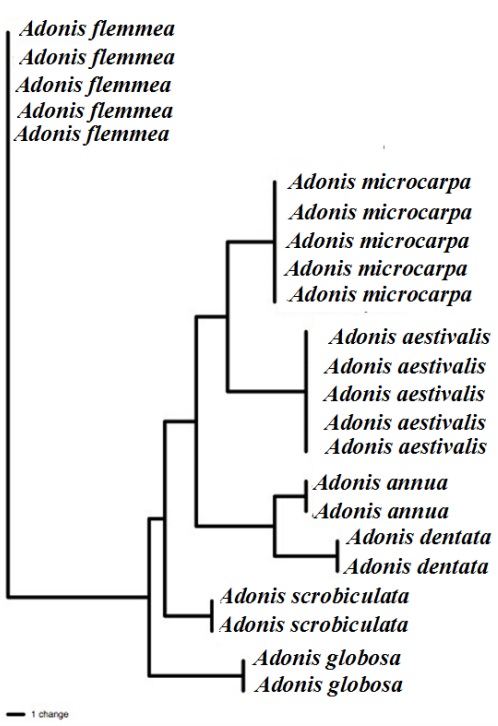

Fig. 2. Maximum parsimony tree of *Adonis* species based on morphological data.

Table 5. Morphological results of Adonis species

Species Characters	*A. scrobiculata*	*A. microcarpa*	*A. globosa*	*A. flammea*	*A. dentata*	*A. annua*	*A. aestivalis*
Length of petal (mm)	7.5	11	8.5	7.5	7.5	7.5	6
Width of petal (mm)	3.5	6	4	3.5	4	4	2.7
Length of sepal (mm)	6	8.5	4	5.5	4.95	5.5	5
Width of sepal (mm)	1.75	3.5	2	3.25	2.25	2.75	2.25
Length of pedicel (mm)	12	12.5	24	23.5	28.5	26	10
Leaf arrangement	alternate-type	alternate-type	alternate-type	whorl-type	alternate-type	alternate-type	alternate-type
Length of internod (mm)	28	26.5	56.5	27	47.5	32	22.5
Length of leaf (mm)	32.5	29	42.5	21.5	35	23.5	23.3
Width of leaf (mm)	23.5	24	24	17	24	16.5	8.15
Flower diameter	17	21	18	17	14.5	18.5	13
Stem branched	Unbranched	branched	Unbranched	Unbranched	unbranched	Unbranched	branched

A. microcarpa and *A. aestivalis*, comprised a separate clade and show close affinity as suggested in Flora Iranica. These two species have branched stem, while the other species are unbranched (Table2).

A. annua and *A. dentata* formed the second clade, followed by *A. scrobiculata* and *A. globosa.* These two species formed separate clades. In general, the species relationship is in agreement with the section treatment in Flora Iranica.

MDS plot of ISSR data (Fig. 3) differentiated the studied *Adonis* species. Moreover, AMOVA revealed significant genetic difference (P = 0.01) among *Adonis* species studied. This indicates significant genetic restructuring in *Adonis* during speciation events. Therefore, ISSR molecular markers can be applied in combination with morphological characters in taxonomy of *Adonis*.

Bayesian tree of ITS sequences (Fig. 4) also revealed that the sect. *Adonis* is differentiated from the sect. *Adonanthe*. Moreover, within the sect. *Adonis* and the studied species, *Adonis flemmea* is much more different from the other taxa. *Adonis microcarpa* showed close affinity to *A. scrobiculata* based on ITS data. Close affinity between *Adonis aestivalis*, *A. microcarpa* and *A.dentata* was also observed in ISSR and morphological data.

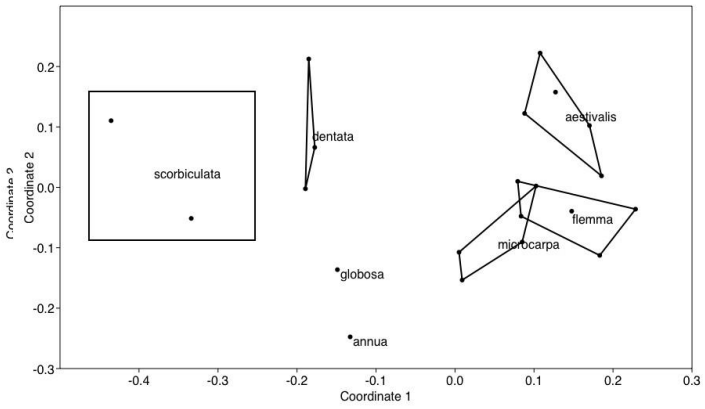

Fig.3. MDS plot of *Adonis* species studied based on ISSR data.

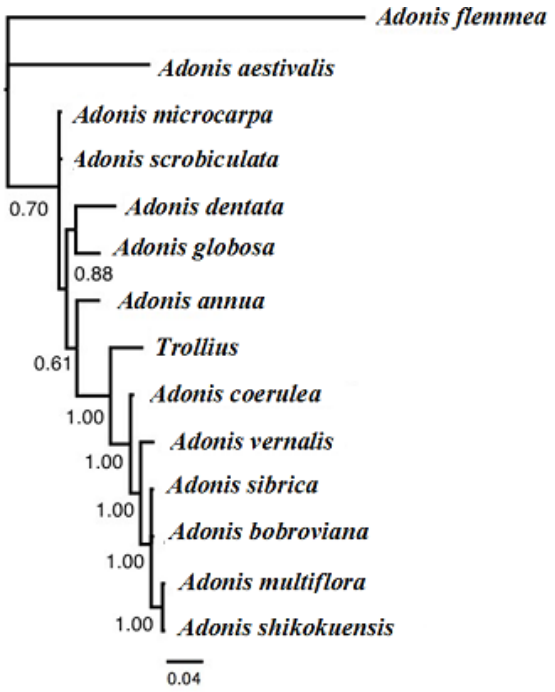

Fig.4. Bayesian tree of *Adonis* species studied based on ITS sequences (Numbers of the branches are clade credibility value).

DISCUSSION

Systematic studied in the genus *Adonis* are based on using different quantitative and qualitative morphological characters liken size of achene, size of leaves, petals, presence versus absence of indumentum, etc. which may show variation in different populations of the same species, and therefore makes species differentiation difficult (SON *et al.*, 2017). In many plant species groups, the same situation is present and therefore species delimitation is difficult. This is also to some degree due to morphological overlap among closely related species or due to absence of clear-cut differentiating characters. Various reasons may bring about such in-discontinuity of morphological characters, like phenotypic plasticity, inter-specific hybridization and introgression (SON *et al.*, 2017). The present study shows that morphological characters like leaf arrangement, leaf length, flower and calyx characteristics are appropriate characters for *Adonis* species differentiation.

The molecular investigations performed (both ISSR markers and ITS sequences) almost delimit the studied *Adonis* species. Therefore, these data are of taxonomic value in the genus. These molecular markers were also almost in agreement with morphological data and produced similar grouping of the species studied. In general, the sect. *Adonis* is monophyletic as evidenced by ITS phylogenetic tree obtained. This is may be due to lack of inter-specific hybridization or introgression as also evidenced by significant Fst values of these species based on ISSR data.

The occurrence of introgression or inter-specific hybridization at both morphological and genetic data can be identified by the occurrence of intermediate phenotypes and species assignment test as evidenced in several plant species (AZIZI *et al.*, 2018; SHEIDAI *et al.*, 2018). Our species assignment test also did not identify any migrants.

CONCLUSIONS

In conclusion, the present study revealed monophyletic nature of sect. *Adonis* and suggest that a combined morphological and molecular approaches can differentiate closely related *Adonis* species.

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MOLEKULARNA FILOGENIJA SECT, *ADONIS* (Genus *Adonis* L., Ranunculaceae)

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Izvod

Ornamentalno važan rod *Adonis* L. (Ranunculaceae) sadrži oko 30 vrsta. u Iranu je 8 vrsta prenoznato. Novi klasifikacioni sistem prepoznaje dva subroda, šest sekcija i šest serija *Adonis*. Diferencijacija vrsta unutar roda je kontrvezralna zbog morfoloških preklapanja u različitim vrstama i velike morfološke varijabilnosti. Ciljevi ovih istraživanja su: 1- Razgraničavanje vrsta unutar Sekc. *Adonis* na osnovu morfoloških i molekualrnih podataka i 2- ispitivanje povezanosti vrsta na osnovu molekualrne filogenije. Multivarjabilna analiza morfoloških i molekualrnih podataka (ITS and ISSR) diferencirala je *Adonis* vrste. PCA biplot analiza morfoloških karakteristika otkriva morfološka svojstva kao što su raspored listova, dužina lista, prečnik cveta, dužina i širina peteljke, dužina kalusa su od taksonomske primena u *Adonis*. Bayesian tree ITS sekvenci otkriva da sekc. *Adonis* je razdvojena od sekc. *Adonanthe*. Na osnovu ITS podataka *A. microcarpa* pokazuje bliski afinitet za *A. scrobiculata*, dok *A. aestivalis*, *A. microcarpa* I *A.dentata* su bliske između sebe.

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