Molecular and micro-morphological evidences of the genus *Cuscuta* in Iran

Received: 31.07.2018 / Accepted: 17.10.2018

Elham Amini: PhD Graduate, Department of Biology, Faculty of Sciences, Gonbad Kavous University, Gonbad, Iran

Fatemeh Nasrollahi: PhD Graduate, Department of Biology, Faculty of Sciences, University of Qom, Qom, Iran

Ali Sattarian✉: Associate Prof., Department of Biology, Faculty of Sciences, Gonbad Kavous University, Gonbad, Iran (sattarian.ali@gmail.com)

Soheila Kor: MSc Graduate, Department of Biology, Faculty of Sciences, Gonbad Kavous University, Gonbad, Iran

Sohrab Boozarpour: Assistant Prof., Department of Biology, Faculty of Sciences, Gonbad Kavous University, Gonbad, Iran

Abstract

Cuscuta is the only parasitic genus in *Convolvulaceae* family. This genus is globally distributed, with most species in the tropics, subtropics, and some in the temperate regions. In this study, the micro-morphological features and molecular evidences of 12 populations from three species of *Cuscuta* (*C. australis*, *C. campestris*, and *C. chinensis*) have been considered. In total, seven quantitative and two qualitative characters of pollen were selected and measured. The most important characters include: shape, ornamentation of tectum, exine thickness and colpus length of the pollen. Based on this study, the seed shape and surface support at least for separation of *C. australis* from other two species. Using nuclear (nrDNA ITS) marker, we reconstructed phylogenetic relationships within three species of *Cuscuta*. This data set was analyzed by phylogenetic methods including Bayesian, Maximum likelihood, and Maximum parsimony. In phylogenetic analyses, all members of three species formed a well-supported clade (PP=1, ML/BS=100/100) and divided into two major clades (A and B). Clade A is composed of specimens of *C. australis*. Two species of *C. campestris* and *C. chinensis* are nested in clade B. Neighbor-Net diagram demonstrated separation of the studied populations. The results showed that, micro-morphological and molecular data provide reliable evidence for separation of these species.

Keywords: *Convolvulaceae*, Neighbor-Net, nrDNA ITS, pollen, seed

شواهد مولکولی و ریزریخت‌شناختی جنس سس در ایران

دریافت: ۱۳۹۷/۰۵/۰۹ / پذیرش: ۱۳۹۷/۰۷/۲۵

الهام امینی: دانش‌آموخته دکتری، گروه زیست شناسی، دانشکده علوم پایه، دانشگاه گنبد کاووس، گنبد کاووس، ایران

فاطمه نصرالهی: دانش‌آموخته دکتری، گروه زیست شناسی، دانشکده علوم پایه، دانشگاه قم، قم، ایران

علی ستاریان✉: دانشیار گروه زیست شناسی، دانشکده علوم پایه، دانشگاه گنبد کاووس، گنبد کاووس، ایران (sattarian.ali@gmail.com)

سهیلا کُر: دانش‌آموخته کارشناسی ارشد، گروه زیست شناسی، دانشکده علوم پایه، دانشگاه گنبد کاووس، گنبد کاووس، ایران

سهراب بوذرپور: استادیار گروه زیست شناسی، دانشکده علوم پایه، دانشگاه گنبد کاووس، گنبد کاووس، ایران

چکیده

Cuscuta تنها جنس انگلی در تیره پیچکیان است. این جنس پراکنش جهانی داشته و اکثر گونه‌های آن در مناطق گرمسیری، نیمه‌گرمسیری و بعضی در مناطق معتدل پراکندگی دارند. در این مطالعه، ویژگی‌های ریزریخت‌شناختی و مولکولی دوازده جمعیت از سه گونه *Cuscuta* (*C. australis*، *C. campestris* و *C. chinensis*) مورد بررسی قرار گرفت تا ارزش تشخیصی آن‌ها ارزیابی شود. در کل، هفت ویژگی کمی و دو ویژگی کیفی در گرده انتخاب شده و مورد اندازه‌گیری قرار گرفتند. مهمترین ویژگی‌ها شامل شکل، تزیینات تکتوم، ضخامت اگزین و طول شیار گرده هستند. براساس این تحقیق، شکل و سطح دانه، جدایی *C. australis* را از دو گونه دیگر مورد تایید قرار داد. در این تحقیق همچنین، با استفاده از نشانگر هسته‌ای (nrDNA ITS)، روابط فیلوژنتیکی در سه گونه از *Cuscuta* بازسازی گردید. داده‌ها توسط آنالیزهای فیلوژنتیکی شامل بیزین، بیشینه درست‌نمایی و بیشینه صرفه‌جویی مورد بررسی قرار گرفتند. در آنالیزهای فیلوژنتیکی همه اعضای این سه گونه یک شاخه با حمایت بسیار بالا (PP=1, ML/BS=100/100) تشکیل دادند و به دو گروه اصلی تفکیک شدند (A و B). شاخه A از نمونه‌های گونه *C. australis* تشکیل شده است و دو گونه *C. campestris* و *C. chinensis* در شاخه B قرار گرفتند. روش شبکه-همسایه، جدایی جمعیت‌های مورد مطالعه را ثابت کرد و نتایج نشان داد که داده‌های ریزریخت‌شناختی و مولکولی، جدایی این گونه‌ها را از هم تایید می‌کنند.

واژه‌های کلیدی: پیچکیان، دانه، شبکه-همسایه، فاصله‌گذار رونویسی شونده درون هسته‌ای، گرده

* مستخرج از پایان‌نامه کارشناسی ارشد نگارنده چهارم به راهنمایی دکتر علی ستاریان و دکتر سهراب بوذرپور ارایه شده به دانشگاه گنبد کاووس

Introduction

Cuscuta L. (*Convolvulaceae*) is a parasitic plant includes more than 150–170 species in the world (Stefanovic *et al.* 2007) with 24 species in the Flora Iranica region (Yuncker & Rechinger 1969). In Flora of Iran, the genus *Cuscuta* is represented by eighteen species includes three subgenera and five sections (Jafari 2017). The *Cuscuta* originates from the Arabic word "Kushkut" which loosely translates as "a tangled wisp of hair" (Austin 1980). It is mainly distributed in temperate regions of the Europe, West Asia, North Africa, and especially North America (Yuncker 1932, Hunziker 1950, Mabberley 1997, Stefanovic *et al.* 2007, Garcia *et al.* 2014) while scattered in north, central and southwest of Iran. Members of *Cuscuta* are characterized by having mostly annual habit, stem parasites, pale stems, slender, herbaceous vines with twining, with no chlorophyll, and no roots (Kuijt 1969, Cronquist 1981, Stewart & Press 1990, Garcia *et al.* 2014, Keskin *et al*. 2017). These species also show 4–5-merous small flowers and tubular-campanulate calyx (Jafari *et al*. 2016).

These stem parasites are attached to the different hosts via haustoria and depend entirely on them to supply nutrients and water (Kuijt 1969, Cronquist 1981, Dawson *et al.* 1994). The specific differences between species of *Cuscuta* are based on the stems, flowers and fruits (Jafari *et al*. 2016). Nearly 15–20 *Cuscuta* sp. are worldwidely agricultural and horticultural pests (Dawson *et al.* 1994, Costea & Tardif 2006). The description of the genus *Cuscuta* was first published in 1700 by Tournefort. In 1932, Yuncker produced a worldwide monograph that divided the genus into three subgenera based on the morphology of stigmas and styles. The first subgenus with one style, *Monogyna* (Englm.) Yunck. is the most distinctive taxa which favors trees and shrubs as hosts. The second subgenus, *Grammica* (Lour.) Yunck., contains two styles, with capitate stigmas and is the most diversified group with the majority of the species. The last subgenus is *Cuscuta*, which is characterized by elongated stigmas and two styles (Yuncker 1932). There is some controversy regarding the family in which to place the genus *Cuscuta*: either in *Convolvulaceae* or an entirely separate family i.e. *Cuscutaceae*. Based on recent molecular studies, *Cuscuta* should remain in *Convolvulaceae* (Garcia & Martin 2007). The study of micro-morphological characters is an important step in the establishment of relationships between the comprising taxa. The pollen morphological analysis has a proved taxonomical significance and is successfully used as an additional criterion for delimitation of the taxa (Welsh *et al*. 2010). For example, Hallier (1893) assigned the genera within the family *Convolvulaceae* to two major groups: "*Echinoconieae*" and "*Psiloconiae*" based on their echinate or psilate exine, respectively. Sengupta (1972) studied 21 *Cuscuta* species and classified two groups according to pollen structures of these species as tricolpate and penta-hexa-colpate. Liao *et al.* (2004) investigated pollen morphology of five taxa of *Cuscuta* by using LM, SEM and TEM that show two distinct pollen types based on ektexine. Study on 11 species in Pakistan displayed that, they are spheroid to oblate and prolate (Perveen & Qaiser 2004). Demir *et al.* (2017) investigated palynological features of taxa belonging to *Cuscuta* in Turkey. The authors displayed that, pollen structure of these taxa were determined prolate, subprolate, perprolate, and prolate-spheroidal, where the apertures of the pollens of these taxa were found scabrate, scabrate-perforate, oscabrate-perforate, reticulate, and ekinate-reticulate. Hamed (2005) studied pollen and seed characters in five *Cuscuta* species growing in Egypt. He suggested that, the genus *Cuscuta* is better looked at as a derived member of *Convolvulaceae* rather than forming a family of its own. Seed exomorphic characters of some *Cuscuta* species were also investigated by some researchers, e.g. *C. pedicellata* Ledeb. and *C. campestris* Yunck. (Lyshede 1992), Chinese *Cuscuta* species (Huang *et al.* 1993), and *C. chinensis* Lam. and *C. gronovii* Willd. (Hamed & Mourad 1994). The seed morphology of eight taxa of *Cuscuta* from Egypt has been studied using light and scanning electron microscopy and a key for the

identification of the investigated taxa based on seed characters was also provided (Abdel Khalik 2006).

Molecular data have been used in numerous studies seeking to clarify the relationships of parasitic taxa despite accelerated rates of sequence evolution (e.g. Nickrent & Starr 1994, Wolfe & Depamphilis1997, Depamphilis *et al.* 1997, Duff & Nickrent 1997,Young *et al.* 1999). These data can also provide supportive and additional criteria for systematic classification of the studied species which was only based on morphological characters, and produced some evidence for identification of infra-specific taxonomic forms (Chase *et al.* 1993). The internal transcribed spacer (ITS) is the region of the 18S-5.8S-26S nuclear ribosomal Cistron (Baldwin *et al.* 1995). The spacers contain the signals needed to process the rRNA transcript (Baldwin 1992, Baldwin *et al.* 1995) and have often been used for inferring phylogeny at the generic and infrageneric levels in plants (e.g. Baldwin 1992, Baldwin *et al.* 1995, Kazempour-Osaloo *et al.* 2003, 2005). Stefanovic & Olmstead (2004) tested the phylogenetic position of *Cuscuta* by three genomes and illustrated the phylogenetic position of this small parasitic plant. Garcia & Martin (2007) presented the phylogenetic study of the subgenus *Cuscuta*, by using nrDNA ITS and chloroplast *trn*L intron sequences. They identified two monophyletic groups within this subgenus. Multiple DNA sequences from plastid (*trn*L-F region and rbcL) as well as nuclear (ITS and 26S rDNA) genomes were used by Costea & Stefanovic (2009) to infer the phylogeny of the *C. californica* complex.

Cuscuta is well-known for its taxonomic complexity resulting from overlapping morphological characters. The objectives of the present study were, therefore, (1) to find micro-morphological evidences in discrimination of closely related species, (2) to use the pollen grains and seed features as a source of diagnostic characters in these species, (3) to investigate molecular properties of this genus in Northern Iran, and (4) to evaluate the affinities and relationships of its three species.

Materials and Methods

In the present study, 12 populations from three species of *Cuscuta* (*C. australis* Hook.f., *C. campestris*, and *C. chinensis*) were obtained from northern parts of Iran during field work from March to July 2016 (Table 1). Some of the collected specimens were dried according to the standard procedures and preserved as herbarium specimens for use in morphological investigations. The identified plants were kept in the Gonbad Kavous University Herbarium (GKUH) (Gonbad Kavous, Iran). The Flora Iranica (Rechinger & Yuncker 1964), and Flora of Iran (Jafari 2017) were used for the identification.

- Morphological methods

The palynological part of this study was made using Light Microscope (LM) and Scanning Electron Microscope (SEM) on pollens of *C. australis*, *C. campestris*, and *C. chinensis*. The pollen samples were obtained mostly from freshly collected herbarium specimens. For LM studies, the samples were acetolyzed following Erdtman's technique (Erdtman 1952). The measurements were based on at least 30 pollen grains per population. These were made with the help of a Nickon light microscope by using a Canon Digital Camera.

For SEM investigation, the pollen grains were transferred directly to double-sided tape affixed stubs and were sputter-coated with gold plates. Photomicrographs were taken with a VEGA//TESCAN-LMU Electron Microscope at an accelerating voltage of 15–22 KW at Research Institute of Razi (Tehran, Iran). The applied terminology based on Punt *et al.* (2007).

Seeds of the three species of *Cuscuta* (*C. australis*, *C. campestris*, and *C. chinensis*) were taken from herbarium specimens. The seeds of every species were carefully examined under the stereomicroscope to ensure the normal size and development, mounted directly on aluminum stubs with the help of two-sided

adhesive tape. After having been coated with a thin layer (ca. 25 nm) of gold, they were analyzed using a VEGA//TESCAN-LMU Electron Microscope at an accelerating voltage of 15–22 KW at Research Institute of Razi (Tehran, Iran). For recording gross morphology and size parameters, the seed type, ornamentation character and color status, at least 10 seeds were assessed by biometric methods. The list of voucher specimens and details of localities are given in table 1.

- Molecular methods

Taxon Sampling: A total of three species of *Cuscuta* and 12 population were chosen as ingroup for nrDNA ITS. Three species of *Cuscuta* (*C. haussknechtii* Yunck., *C. japonica* Choisy, and *C. monogyna* Schmidt ex Engelm) were chosen as outgroups following previous molecular phylogenetic studies (Garcia & Martin 2007). A list of all the taxa used in this study and the sources, voucher information and GenBank accession numbers are also given in table 1.

DNA extraction, PCR and sequencing: Total genomic DNA was extracted from dried leaf materials deposited in Gonbad Kavous University Herbarium (GKUH), using Kit method. The nrDNA ITS region was amplified using the primers ITS5m (Sang *et al*. 1995) and ITS4 (White *et al.* 1990). PCR amplification of the DNA regions followed procedures described in detail by Naderisafar *et al.* (2014). The quality of PCR products was checked by electrophoresis in 1% agarose gels in 1 × TAE (pH=8) buffer and were photographed with a UV gel documentation system (UVI Tec, Cambridge, UK). PCR products along with the same primers were sent for Sanger sequencing at Macrogen (Seoul, South Korea) through Pishgam Inc. (Tehran, Iran).

Sequence alignment: Each of the single datasets was aligned using the web-based Ver. of MUSCLE (Edgar 2004; at http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters followed by manual adjustment. The alignment of datasets required the introduction of numerous single- and multiple-based indels (insertions/deletions). Positions of indels were treated as missing data for all datasets.

- Phylogenetic inferences

Parsimony method: Maximum parsimony (MP) analyses were conducted using PAUP* Ver. 4.0a157 (Swofford 2002). The heuristic search option was employed for each dataset, using tree bisection-reconnection (TBR) branch swapping, with 1000 replications of random addition sequence and an automatic increase in the maximum number of trees. Uninformative characters were excluded from the analyses. Branch support values (MPBS) were estimated using a full heuristic search with 1000 bootstrap replicates (Felsenstein 1985) each with simple addition sequence.

Likelihood method: Maximum likelihood analysis (ML) was performed on each dataset, using RAxML Ver. 8.2.10 (Stamatakis 2014), as implemented in the CIPRES Science Gateway. The model of evolution employed for each data-set is the same as that of Bayesian analyses. Bootstrap values (MLBS) were calculated in RAxML based on 1000 replicates with one search replicate per bootstrap replicate. Overall, mean p-distance for each dataset was computed using MEGA7 (Kumar *et al*. 2016).

- Bayesian inference

For Bayesian inference (BI) analyses, models of sequence evolution were selected using the program MrModeltest Ver. 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada & Buckley 2004). This program indicated a SYM+G model for nrDNA ITS, as the best models for nucleotide substitution. BI analyses were performed using MrBayes Ver. 3.2 (Ronquist *et al.* 2012) on the CIPRES Science Gateway (Cyber Infrastructure for Phylogenetic Research Cluster) (Miller *et al.* 2010, https://www.phylo.org) for the datasets.

Bayesian analyses were performed, with default priors (uniform priors) and the best-fit model of sequence evolution for each dataset, with two runs of ten million generations and four simultaneous chains (one cold and three heated with a heating parameter of 0.2) by saving trees every 100 generations. The trees sampled after discarding 25% as "burn-in" were collected to build a 50% majority rule consensus phylogram were used to calculate posterior probability values (PP). Tree visualization was drawn using TreeView Ver. 1.6.6 (Page 2001).

- Phylogenetic networks

Neighbor-Net (NN) a distance-based network construction method (Bryant & Moulton2004) was implemented in SPLITS TREE4, Ver. 4.14.4 (Huson 1998) using a Dice dissimilarity matrix. The ITS matrice was modified prior to analysis by excluding outgroups.

Results

Cuscuta is one of the most important genus of *Convolvulaceae*. Three species and 12 populations of this genus have been studied in terms of pollen and seed micro-morphological and molecular phylogeny. Micrographs of pollen and seeds characteristics obtained by SEM are shown in figures 1 and 4. They are used to define the characters which help distinguish between the species.

- Pollen morphology

The pollen grains of the studied species revealed variations and separated three species of *Cuscuta*. All palynological structures and measurements for the examined species concerning pollen type from polar view, polar (P) and equatorial (E) measurements, P/E ratio, colpus width, colpus length, colpus distance**,** pollen shape, and tectum ornamentation are shown in table 2. Selected SEM micrographs of the pollens and their surfaces are shown in figure 1. Generally, exine thickness, colpus lenght, pollen shape and ornamentation were found useful in separating three species. Polar axis (P) length of pollen grains ranging from the smallest size for *C. australis* (22.32 µm) to the largest size for *C. campestris* (43.62 µm). Equatorial axis (E) length of pollen grains ranged from the smallest size in *C. australis* (24.66 µm) to the largest size in *C. campestris* (43.53 µm). The shape classes are based on the ratio between the length of polar axis (P) and equatorial diameter (E). The P/E ratio ranged from 0.91–0.97 µm, therefore, shows the pollen grains are prolate to circular. The smallest and largest exine thickness is observed in *C. australis* (1.64 µm) and *C. campestris* (2.89), respectively. Tectum ornamentation is coarsely reticulate in *C. australis* (Fig. 1F) and finely reticulate in *C. chinensis* (Fig. 1B) and *C. campestris* (Fig. 1C).

Table 1. List of *Cuscuta* species used in this study along with localities and vouchers

Taxon	Collection data (all samples are from Iran)	GenBank accession No. ITS
C. australis	Gilan province: Chaf, Soheila Kor 201665 (GKUH)	MH633914.1
C. australis	Gilan province: Langerud, Soheila Kor 201666 (GKUH)	MH633913.1
C. australis	Gilan province: Talesh, Soheila Kor 201668 (GKUH)	MH633912.1
C. australis	Gilan province: Rudsar, Soheila Kor 201669 (GKUH)	MH633911.1
C. campestris	Gilan province: Rasht, Soheila Kor 2016611 (GKUH)	MH633910.1
C. campestris	Golestan province: Golidagh, Soheila Kor 2016613 (GKUH)	MH633909.1
C. campestris	Golestan province: Bandar Torkman, Soheila Kor 2016614 (GKUH)	MH633908.1
C. campestris	Mazandaran province: Galugah, Soheila Kor 2016615 (GKUH)	MH633907.1
C. chinensis	Golestan province: Gonbad Kavous, Soheila Kor 2016616 (GKUH)	MH633906.1
C. chinensis	Golestan province: Golestan forest, Soheila Kor 2016618 (GKUH)	LC457028
C. chinensis	Golestan province: Incheborun, Soheila Kor 2016619 (GKUH)	LC457029
C. chinensis	Gilan province: Darkumeh, Soheila Kor 2016620 (GKUH)	LC457030
C. haussknechtii	Genbank	DQ924580.1
C. japonica	Genbank	DQ924571.1
C. monogyna	Genbank	DQ924569.1

GKUH: Gonbad Kavous University Herbarium

Table 2. Pollen morphological characters for the examined *Cuscuta* species

Taxon	Polar axis (µm)	Equatorial axis (µm)	P/E (µm)	Colpus width (µm)	Colpus length (µm)	Colpus distance (µm)	Exine thickness (µm)	Shape	Tectum ornament-ation
C. australis	47.66±0.25	29.63±0.15	1.60	2.78±0.31	12.04±0.21	14.14±0.11	1.87±0.13	Prolate	Scabrate
C. australis	45.43±0.32	27.36±0.45	1.66	3.16±0.35	14.24±0.25	16.21±0.12	1.94±0.16	Prolate	Scabrate
C. australis	48.32±0.35	24.66±0.23	1.95	3.18±0.21	13.23±0.15	13.25±0.15	1.77±0.18	Prolate	Scabrate
C. australis	46.27±0.22	27.25±0.25	1.69	2.58±0.41	12.44±0.25	14.33±0.21	1.64±0.26	Prolate	Scabrate
C. campestris	40.67±0.24	42.56±0.35	0.95	4.58±0.45	17.28±0.18	15.04±0.25	2.89±0.16	Circular	Reticulate-Echinate
C. campestris	43.62±0.35	45.36±0.28	0.95	4.28±0.41	19.12±0.22	16.17±0.31	2.37±0.25	Circular	Reticulate-Echinate
C. campestris	41.47±0.21	43.53±0.21	0.95	4.18±0.31	18.04±0.15	15.14±0.35	2.67±0.36	Circular	Reticulate-Echinate
C. campestris	40.37±0.34	42.46±0.25	0.95	3.98±0.21	19.34±0.25	16.14±0.21	2.43±0.26	Circular	Reticulate-Echinate
C. chinensis	39.26±0.24	40.26±0.35	0.97	3.78±0.34	20.24±0.45	14.24±0.24	2.57±0.16	Circular	Reticulate-Echinate
C. chinensis	38.37±0.34	39.42±0.25	0.97	3.83±0.41	22.04±0.35	15.14±0.33	2.17±0.36	Circular	Reticulate-Echinate
C. chinensis	39.17±0.37	40.65±0.24	0.97	3.73±0.38	24.34±0.65	16.24±0.11	2.39±0.24	Circular	Reticulate-Echinate
C. chinensis	40.33±0.34	42.26±0.35	0.95	3.68 ± 0.35	19.54±0.15	13.14±0.21	2.62±0.16	Circular	Reticulate-Echinate

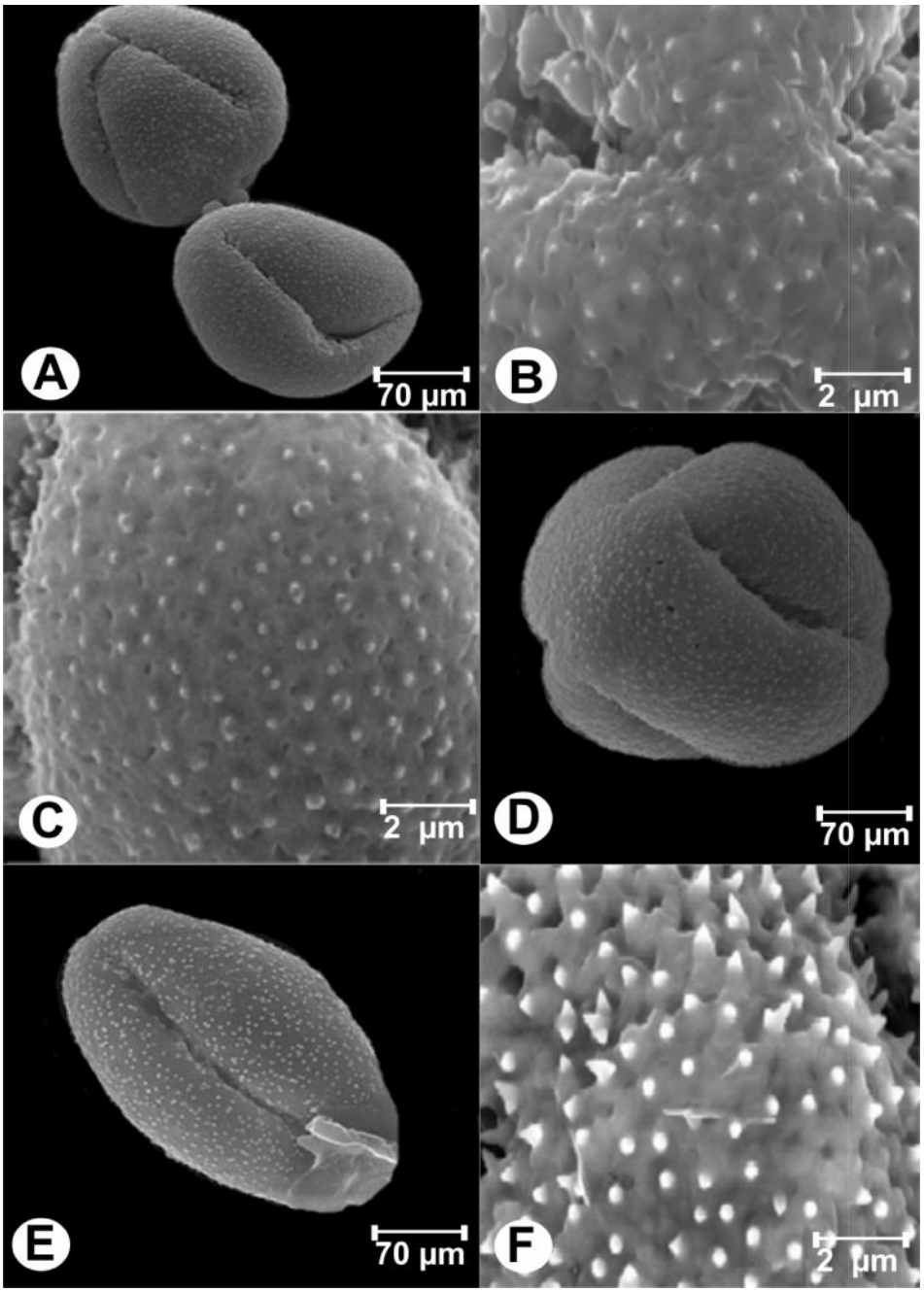

Fig. 1. Scanning electron micrographs (SEM) of pollen surface in *Cuscuta campestris*, *C. chinensis*, and *C. australis*. For each taxon the first micrograph shows the outline of the pollen indicating its general shape, and the second micrograph is a close view of the pollen surface. A, B. *Cuscuta campestris*, C, D. *C. chinensis*, and E, F *C. australis*.

In order to define the diagnostic value of pollen grains in species delimitations in studied *Cuscuta* species, cluster analysis by Ward's method was carried out based on nine qualitative and quantitative features (Fig. 2).

Ward's dendrogram showed two main clusters (Fig. 2). First cluster composed of *Cuscuta australis*, and the second cluster composed of two subsets included *C. chinensis*, and *C. campestris* populations.

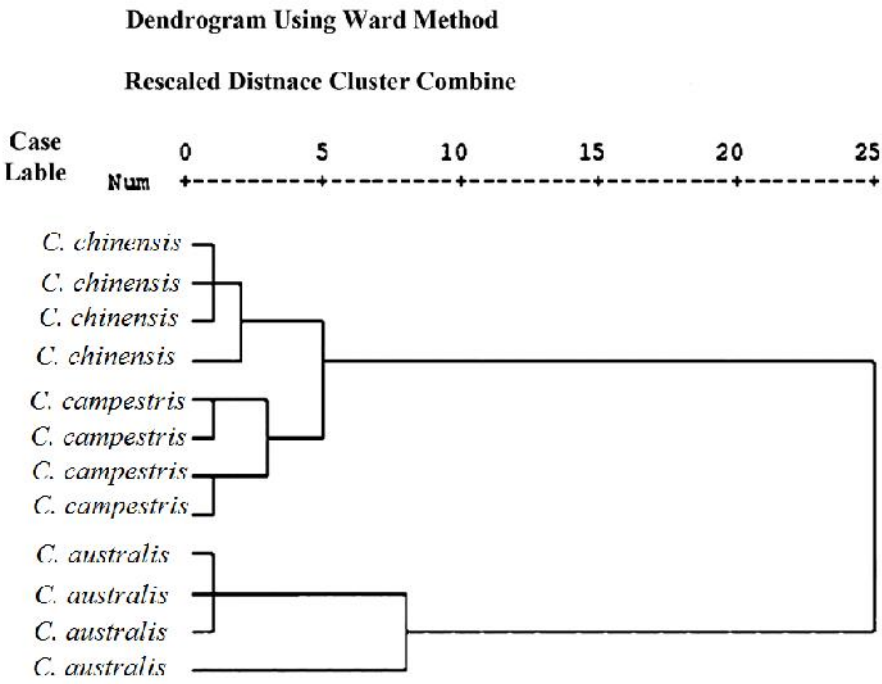

Fig. 2. Cluster analysis (Ward's method) based on pollen features of *Cuscuta.*

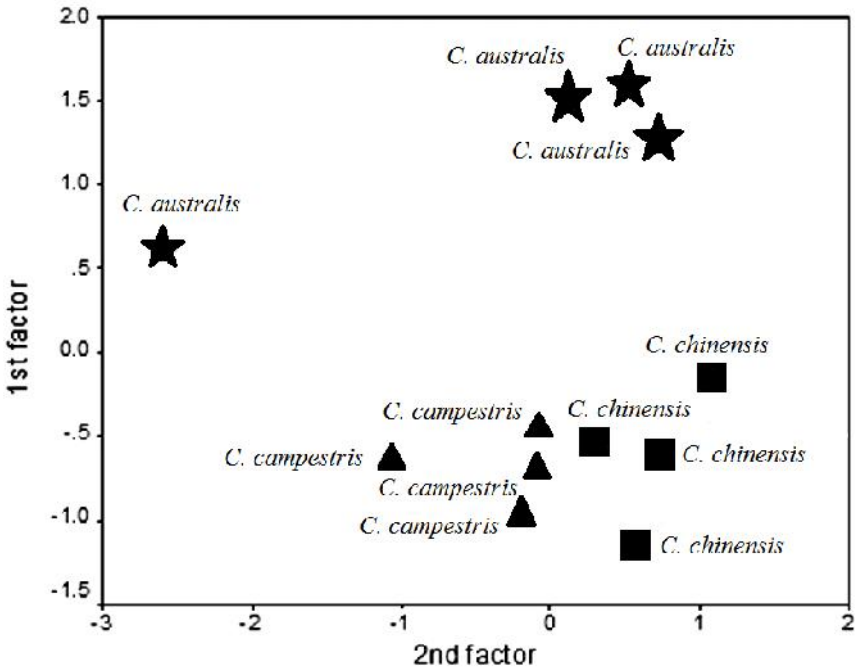

Fig. 3. PCO plot of *Cuscuta* species based on observed pollen data.

Factor analysis revealed that, there were two factors provided more than 72% of total observed variation in studied pollen grains. By studying the component matrix for each factor, it was evident that, shape and ornamentation of tectum are most important features in the first factor and exine thickness and colpus length, are most significant in the second factor. PCO confirmed the results of cluster analysis by Ward's method based on qualitative and quantitative features of pollen grains (Fig. 3).

- Seed characteristics

Values of six quantitative and qualitative seed traits have been observed and measured in three *Cuscuta* species (Table 3). SEM photographs for each species, showing the seed character variations, are given in figure 4. Seeds are generally almond, with various degrees of deviation. However, circular seeds were also occasionally observed among some examined species. The longest length of the seeds is seen 3.48 mm in *C. chinensis*, and shortest width is found 1.37 mm in *C. australis* (Table 3). In terms of exomorphology, seed surface is generally regulate in *C. australis* (Fig. 4F), polygonal in *C. chinensis* (Fig. 4B) and *C. campestris* (Fig. 4C). The anticlinal walls are observed coarsely undulate in *C. australis* (Fig. 4F), and deeply undulate in both *C. chinensis* and *C. campestris* (Figs 4B, C).

Table 3. Some diagnostic seed micro-morphological features in *Cuscuta* species

Taxon	Length (mm)	Width (mm)	Length/width	Shape	Anticlinal wall	Sculpturing
C. australis	2.18±0.01	1.47±0.04	1.48	Circular	Coarsely undulate	Regulate
C. australis	2.05±0.01	1.37±0.04	1.49	Circular	Coarsely undulate	Regulate
C. australis	2.20±0.02	1.58±0.04	1.39	Circular	Coarsely undulate	Regulate
C. australis	2.25±0.02	1.47±0.06	1.53	Circular	Coarsely undulate	Regulate
C. campestris	3.15±0.02	2.87±0.06	1.09	Almond	Deeply undulate	Polygonal
C. campestris	3.30±0.02	2.18±0.06	1.51	Almond	Deeply undulate	Polygonal
C. campestris	3.38±0.01	2.38±0.06	1.42	Almond	Deeply undulate	Polygonal
C. campestris	3.40±0.01	2.57±0.06	1.32	Almond	Deeply undulate	Polygonal
C. chinensis	3.35±0.01	2.48±0.06	1.35	Almond	Deeply undulate	Polygonal
C. chinensis	3.45±0.07	2.86±0.02	1.20	Almond	Deeply undulate	Polygonal
C. chinensis	3.48±0.01	2.65±0.01	1.31	Almond	Deeply undulate	Polygonal
C. chinensis	3.28±0.01	2.79±0.01	1.17	Almond	Deeply undulate	Polygonal

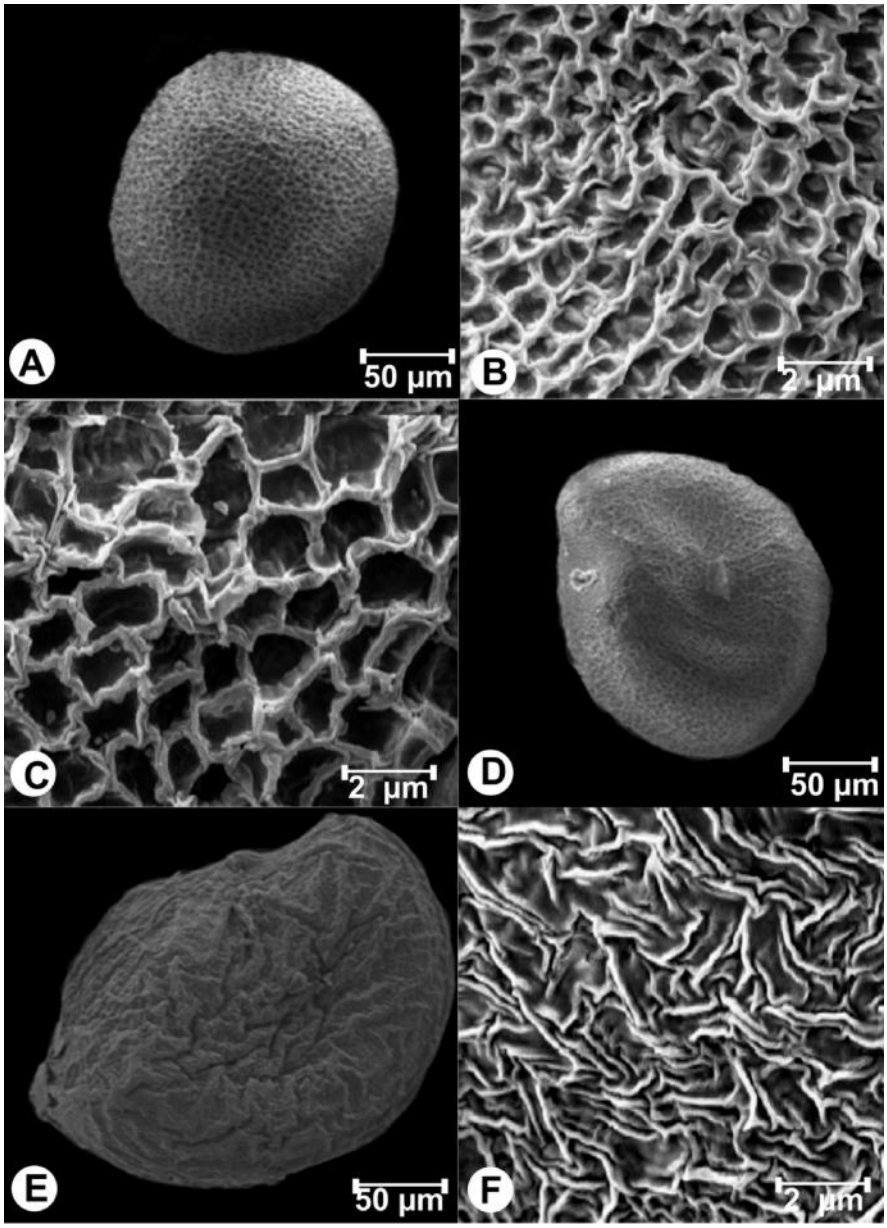

Fig. 4. Scanning electron micrographs (SEM) of seed surface in *C. campestris*, *C. chinensis*, and *C. australis*. For each taxon, the first micrograph shows the outline of seed indicating its general shape, and the second micrograph is a close view of seed surface. A, B. *Cuscuta campestris*, C, D. *C. chinensis*, and E, F. *C. australis*.

- Phylogenetic analysis

Detailed information about alignment characteristics, selected model of nucleotide substitution, as well as tree statistics from the single analysis of the nrDNA ITS region, are summarized in table 4. The aligned nrDNA ITS matrix comprises 658 characters. The parsimony and Bayesian analyses of the nrDNA ITS produced congruent trees and gave similar results. All members of this genus form a well-supported clade (PP=1, MP/BS=100) and divided into two well-supported major clades (Fig. 5). Clade A is composed of species of *C. australis.* Two species of *C. campestris* and *C. chinensis* were nested in clade B. This clade (B) further forms two subclades including B1 and B2.

Table 4. Dataset and tree statistics from single analysis of the nuclear region

Total sample	ITS
Number of sequences	15
Number of ingroup sequences	12
Alignment length [bp]	658
Number of parsimony-informative characters	145
Number of MPTs	92
Length of MPTs	119
Consistency index (CI)	0.872
Retention index (RI)	0.740
Evolutionary model selected (under AIC)	SYM + G

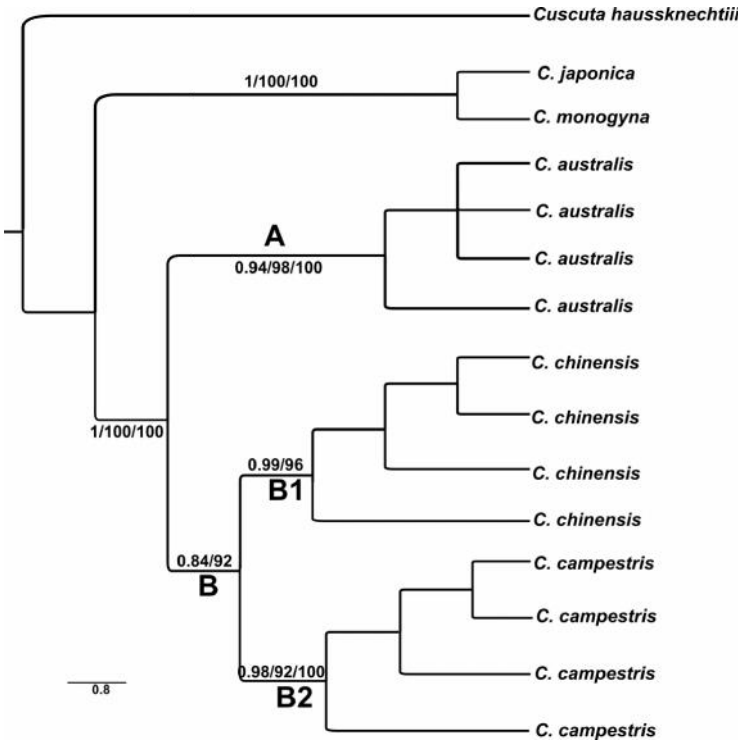

Fig. 5. 50% majority rule consensus tree resulting from the Bayesian phylogenetic analysis of the nrDNA ITS dataset. Numbers of the branches are posterior probability (PP) from the BI and bootstrap support (BS) values from a MP and ML analysis, respectively (values <50 % were not shown).

- Phylogenetic networks

The splits graph shows major internal network structure, indicating reticulation. Correlation between geographical and genetic distance of the studied populations was studied on the basis of Podani (2000). The groups formed in the splits graph are readily correlated to the clades recovered in the phylogenies.

Populations of *C. australis* (9–12) are distinct and stand separately from the other populations at a major distance. Populations of *C. chinensis*, and *C. campestris* (1–8) showed more genetic affinity and placed close to each other.

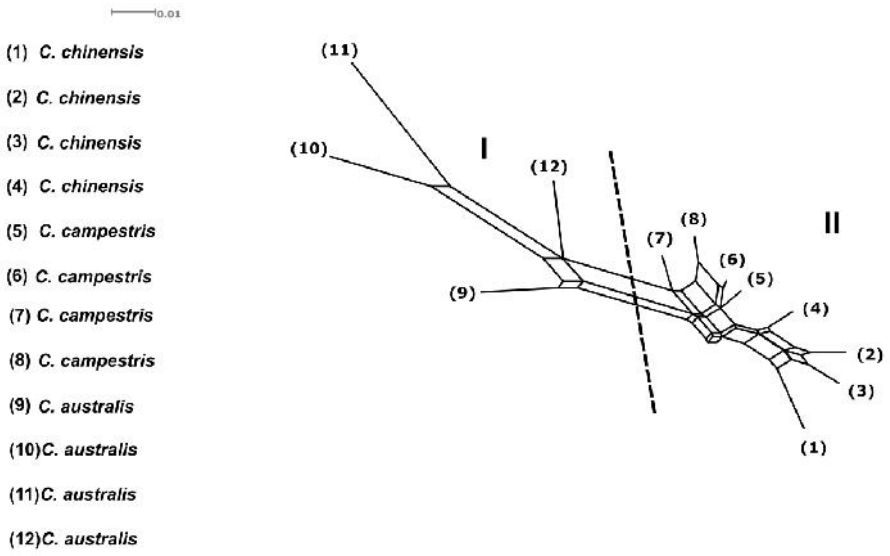

Fig. 6. Splits graph for ITS sequences of *Cuscuta*. Two major groups were recovered (lineage I and II).

Discussion

The taxonomic position of *Cuscuta* has been discussed from the past due to the existence of several specialized traits that are suitable for parasitic life, including the reduction of chlorophyll content and scale leaves. The reduction of morphological traits, the consistency of these traits among species and the presence of diversity at the lower level, is one of the most important problems in determining the taxonomic boundaries in this genus. Because of little attention in previous micro-morphological and phylogenetic studies, this study represents the first general investigation of the *Cuscuta* in Iran. The morphological pollen structures were found different from each other and these properties were found to be important for taxonomy of *Cuscuta* (Table 2). The genus is extremely variable in their pollen characters. Pollen results have indicated that, *C. chinensis* had the largest, while *C. australis* had the smallest pollen grains (Table 2). Sengupta (1972) worked out an important study on the pollen morphology of *Convolvulaceae*. His study pointed out that, the pollen grains of 19 species of *Cuscuta* are tricolpate where some of accessions are tetracolpate and pantocolpate which is in accordance with our results. Welsh *et al.* (2010) stated that, the pollen structures of *Cuscuta* were heteromorphic and almost 95% of the studied species were 3-zonocolpate. Moore *et al*. (1991) reported that, the pantocolpate condition of some species, containing *Cuscuta* that are normally trizonocolpate, is thought to be associated with meiotic irregularities or increasing ploidy levels, and sometimes with hybridization. Our study is also in accordance with Perveen & Qaiser (2004) that shape of Pakistani species are spheroidal to oblate, and prolate and tectum with reticulate, regulate and scabrate processes. Also, based on study of Liao *et al.* (2004), two distinct pollen types of *Cuscuta* spp. in Taiwan are recognized. Type 1 (including *C. australis*, *C. campestris*, and *C. chinensis*), is small-sized and has colpus with granules, and ektexine finely reticulate; while type 2 (including *C. japonica*), is medium-sized, and has colpus with granules, scabrate processes on surface of granule and reticulate ektexine. Results of palynological studies on different species of *Cuscuta* verified importance of pollen traits for distinguishing this taxa. As it is visible in Ward's dendrogram, species are separated from each other by their pollen features.

The present study about seed is in accordance with the findings of Abdel Khalik (2006) that, the seed surface separates all the species clearly. *Cuscuta australis* has regulate sculpture, where *C. chinensis* and

C. campestris have polygonal sculpture. Abdel Khalik (2006) demonstrated that, subgenera *Grammica* and *Cuscuta* have the same seed and are far from subgenus *Monogyna*. This probably represents apomorphic seed characteristics in these two subgenera (in consistence with finding of Hamed 2005).

In general, it can be concluded that, reproductive traits such as pollen and seed traits have achieved the end of their evolution and are valuable in systematic studies. This could be due to the parasitic life and invasive nature of this plant to occupy new areas of growth.

Plant molecular studies greatly advanced in the recent years and molecular phylogenetic investigation has dramatically re-shaped our views of organismal relationships (Soltis & Soltis 2000). Nuclear molecular technique has been successfully applied for research of infraspecific variations in different genera (Sheidai *et al.* 2013, 2014). Therefore, we decided to use the molecular approach for investigation of infra-specific variations between *Cuscuta* species. Phylogenetic analyses indicated the monophyly of *Cuscuta* with strongly support (PP=1.00, ML/BS=100/100) and divided into two major clades. Our result is also in concordance with Stefanovic & Olmstead (2004). In their sampling, two species of *Cuscuta*, *viz. C. japonica* (subgenus *Monogyna*), and *C. europaea* (subgenus *Cuscuta*), were identified as monophyletic. These species chosen as place-holders for the genus, because they exemplify the morphological (united style versus bifid style) and physiological (hemiparasite versus holoparasite) diversity within the genus. All of the analyses of Costea & Stefanovic (2009) show the *C. californica* complex to be a strongly supported monophyletic group and within this complex, four major lineages can be delimited based on a combination of their individual strong support. Even though, we confirm the monophyly of the genus *Cuscuta* relationships among some species in clade B, were poorly resolved and form a polytomy. In fact, sequence divergence among these taxa was generally low, resulting in a lack of phylogenetic resolution. Molecular study (*rbcl* and nrLSU) conducted by Garcia *et al.* (2014), did not confirm any close relationship between *C. chinensis* and *C. campestris*. Although, important factors that could be responsible for this incongruence are: differences in number of species and choice of molecular markers. In a study carried out by Garcia & Martin (2007), the position of some taxon is not resolved and arises from the polytomy of a clade. They believed that, factors influencing the taxonomic difficulty of many species in the subgenus *Cuscuta*, include lack of morphological characters, parallelism and gene flow between closely- and not-so-closely related species. Also, it is evident that, reliance on a single data set may result in inadequate resolution or an incorrect picture of phylogenetic relationships (Soltis & Soltis 2000). Therefore, it is necessary to use chloroplast markers to better resolve the relationships between the populations of each species.

Neighbor-Net (NN), a distance-based network construction method, allows for graphical representation of conflicting phylogenetic signals and interpretation of evolutionary histories which are not tree-like (Bryant & Moulton 2004). Neighbor-Net split graphs have been used with varying success to detect reticulate history (Carine *et al*. 2007, Frajman & Oxelman 2007, Grimm & Denk 2008, Weiss-schneeweiss *et al*. 2008, Ramdhani *et al.* 2010). In this study, the Neighbor-Net diagram demonstrated complete separation of the studied populations within the network, supporting the phylogenetic results. The splits graph shows extensive internal network structure, indicating reticulation. The groups formed in the splits graph are readily correlated to the clades recovered in the phylogenies. We use the term “lineage” to refer to groups of specimens in the NN trees and “clade” to refer to groups in the phylogenies (Fig. 6).

The ITS splits graph revealed two main groups (Fig. 6). Lineage “I” correlates to clade “A” and is composed of populations of *C. australis*. Lineage “II”

includes the populations of *C. chinensis* and *C. campestris*, correlates to clade "B" (Fig. 5).

Conclusion

Cuscuta is a parasitic genus due to the existence of several specialized characters; the taxonomic position of it has been unresolved. Present study was carried out to provide additional evidence for taxonomists to help separate these three species. These taxa differ in important micro-morphological and molecular characteristics. It is necessary to use chloroplast markers to better resolve the relationships between the populations of each species.

Acknowledgment

The authors would like to thank Razi Institute (Tehran, Iran) staff for their assistance in scanning electron microscopy, and all of persons who helped us in this research work.

References

Abdel Khalik, K. & Van der Maesen, L.J.G. 2002. Seed morphology of some tribes of *Brassicaceae* (implications for taxonomy and species identification for the flora of Egypt). Blumea 47: 363–383.

Austin, D.F. 1980. Studies of the Florida *Convolvulaceae* III. *Cuscuta*. Florida Scientist 43: 294–302.

Baldwin, B.G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the *Compositae*. Molecular Phylogenetics and Evolution 1: 3–16.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S. & Donoghue, M.J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Annals of the Missouri Botanical Garden 82: 247–277.

Bryant, D. & Moulton, V. 2004. Neighbor-Net: An agglomerative method for the construction of phylogenetic networks. Molecular Biology and Evolution 21: 255–265.

Carine, M.A., Robba, L., Little, R., Russel, S. & Guerra, A.S. 2007. Molecular and morphological evidence for hybridization between endemic Canary Island *Convolvulu*s. Botanical Journal of the Linnean Society 154: 187–204.

Chase, M.W., Soltis, D.E., Olmstead, R.G. *et al*. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene rbcL. Annals of the Missouri Botanical Garden 80: 528–580.

Costea, M. & Tardif, F.J. 2006. Biology of Canadian weeds. *Cuscuta campestris* Yuncker, *C. gronovii* Willd. ex Schult., *C. umbrosa* Beyr. ex Hook., *C. epithymum* (L.) L., and *C. epilinum* Weihe. Canadian Journal of Plant Science 86: 293–316.

Cronquist, A. 1981. An Integrated System of Classification of Flowering Plants. Columbia University Press, New York, USA.

Dawson, J.H., Musselman, L.J.,Wolswinkel, P. & Dörr, I. 1994. Biology and control of *Cuscuta*. Weed Science Society of America 6: 265–317.

Demir, I., Kaya, I., Benli, M. & Altinoglu, M.K. 2017. Investigation of Palinological features of taxa belonging to the genus *Cuscuta* distributed in Turkey. International Journal of Agriculture and Biology 19: 746–750.

Depamphilis, C.W., Young, N.D. & Wolfe, A.D. 1997. Evolution of plastid gene rps2 in a lineage of hemiparasitic and holoparasitic plants: Many losses of photosynthesis and complex patterns of rate variation. Proceedings of the National Academy of Sciences of the United States of America 94: 7367–7372.

Duff, R.J. & Nickrent, D.L. 1997. Characterization of mitochondrial small-subunit ribosomal RNAs

from holoparasitic plants. Journal of Molecular Evolution45: 631–639.

Edgar, R.C. 2004. Muscle: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792–1797.

Erdtman, G. 1952. Pollen Morphology and Plant Taxonomy. Angiosperms. Chronica Botanica Co., Waltham, Massachusettes. Copenhagen.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791.

Frajman, B. & Oxelman, B. 2007. Reticulate phylogenetics and phytogeographical structure of *Heliosperma* (*Sileneae*, *Caryophyllaceae*) inferred from chloroplast and nuclear DNA sequences. Molecular Phylogenetics and Evolution43: 140–155.

Garcia, M.A., Costea, M., Kuzmina, M. & Stefanovic, S. 2014. Phylogeny, character evolution, and biogeography of *Cuscuta* (dodders; *Convolvulaceae*) inferred from coding plastid and nuclear sequences. American Journal of Botany101: 670–690.

Garcia, M.A. & Martin, M.P. 2007. Phylogeny of *Cuscuta* subgenus *Cuscuta* (*Convolvulaceae*) based on nrDNA ITS and chloroplast trnL intron sequences. Systematic Botany 32: 899–916.

Grimm, G.W. & Denk, T. 2008. ITS evolution in *Platanus* (*Platanaceae*): Homoeologues, pseudogenes and ancient hybridization. Annals of Botany101: 403–419.

Hallier, H. 1893. Versucheinernaturlichengliederung der Convolvulaceen auf morphologischer und anatomischergrundlage. BotanischeJahrbucher fur Systematik16:
453–591.

Hamed, A.K. 2005. Pollen and seed characters of certain *Cuscuta* species growing in Egypt with a reference to a taxonomic treatment of the genus. International Journal of Agriculture and Biology 7: 325–332.

Hamed, K.A. & Mourad, M.M. 1994. Seed exomorphic and anatomical characters of some species of *Convolvulaceae*. Egyptian Journal of Botany 34: 1–16.

Huang, J.Z., Li, Y.H. & Zhang, Y.Y.1993. Surface ultrastructure of seeds of Chinese *Cuscuta*. Acta Phytotaxonomica Sinica 31: 261–265.

Hunziker, A.T. 1950. Las especies de *Cuscuta* (*Convolvulaceae*) de Argentina y Uruguay. Revista de la Facultad de Ciencias Exactas Fisicas y Naturales 12: 1101–1202.

Huson, D.H. 1998. Splits Tree: A program for analyzing and visualizing evolutionary data. Bioinformatics 14: 68–73.

Jafari, E., Assadi, M. & Ghanbarian, G.A. 2016. A revision of *Cuscutaceae* family in Iran. Iranian Journal of Botany 22: 23–29.

Jafari, E. 2017. *Cuscutaceae*. Pp. 3–51. *In*: Assadi, M. *et al*. (eds). Flora of Iran. Research Institute of Forests and Rangeland, Tehran.

Kazempour-Osaloo, S., Maassoumi, A.A. & Murakami, N. 2003. Molecular systematics of the genus *Astragalus* L. (*Fabaceae*): Phylogenetic analyses of nuclear ribosomal DNA internal transcribed spacers and chloroplast gene ndhF sequences. Plant Systematics and Evolution 242: 1–32.

Kazempour-Osaloo, S., Maassoumi, A.A. & Murakami, N. 2005. Molecular systematics of the Old World *Astragalus* (*Fabaceae*) as inferred from nrDNA ITS sequence data. Brittonia 57: 367–381.

Keskin, F., Kaya, I., Usta, M., Demir, I., Hioglu, H.M.S. & Nemli, Y. 2017. Molecular cloning and sequence analysis of its region of nuclear ribosomal DNA for species identification in dodders (*Cuscuta*; *Convolvulaceae*). International

Journal of Agriculture and Biology 19: 1447–1451.

Kuijt, J. 1969. The Biology of Parasitic Flowering Plants. University of California Press, Berkeley, California, USA.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis Ver. 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870–1874.

Liao, G.I., Chen, M.Y. & Kuoh, C.S. 2005. Pollen morphology of *Cuscuta* (*Convolvulaceae*) in Taiwan. Botanical Bulletin of Academia Sinica 46: 75–81.

Lyshede, O.B. 1992. Studies on mature seeds of *Cuscuta pedicellata* and *C. campestris* by electron microscopy. Annals of Botany 69: 365–371.

Miller, M.A., Pfeiffer, W. & Schwartz, T. 2010. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, Louisiana. Piscataway, IEEE, pp. 45–52.

Moore, P.D., Webb, J.A. & Collinson, M.E. 1991. Pollen Analysis. Blackwell Scientific Publications, London.

Naderisafar, K., Kazempour-Osaloo, S., Maassoumi, A.A. & Zarre, Sh. 2014. Molecular phylogeny of *Astragalus* section *Anthylloidei* (*Fabaceae*) inferred from nrDNA ITS and plastid *rpl*32-*trn*$L_{(UAG)}$ sequence data. Turkish Journal of Botany 38: 637–652.

Nickrent, D.L. & Starr, E.M. 1994. High rates of nucleotide substitution in nuclear small-subunit (18S) rRNA from holoparasitic flowering plants. Journal of Molecular Evolution 39: 62–70.

Nylander, J.A.A. 2004. MrModeltest Ver. 2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Page, D.M. 2001. TreeView (Win 32) Ver. 1.6.6. Available: http:// taxonomy.zoology.gla.ac.uk/rod/treeview.html.

Perveen, A. & Qaiser, M. 2004. Pollen flora of Pakistan-XLI. *Cuscutaceae*. Pakistan Journal of Botany 36: 475–480.

Podani, J. 2000. Introduction to the Exploration of Multivariate Data. Backhuyes, Leiden, 407 pp.

Posada, D. & Buckley, T.R. 2004. Model selection and model averaging in phylogenetics: Advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. Systematic Biology 53: 793–808.

Punt, W., Hoen, P.P., Blackmore, S., Nilsson, S. & Thomas, A.L. 2007. Glossary of pollen and spore terminology. Review of Palaebotany and Palynology 143: 1–81.

Ramdhani, S., Cowling, R.M. & Barker, N.P. 2010. Phylogeography of *Schotia* (*Fabaceae*): Recent evolutionary processes in an ancient thicket biome lineage. International Journal of Plant Sciences 171: 626–640.

Rechinger, K.H. & Yuncker, T.G. 1964. *Cuscutaceae*. Pp. 1–16. *In*: Rechinger, K.H. (ed.), Flora Iranica, Vol. 8. AkademischeDruck-u.-Verlagsanstalt. Graz.

Ronquist, F., Teslenko, M., Vander mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. 2012. MrBayes Vol. 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61: 539–542.

Sang, T., Crawford, D. J. & Stuessy, T. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implication for biogeography and concerted evolution. Proceedings of the National Academy of Sciences of the United States of America 92: 6813–6817.

Sengupta, S. 1972. On the pollen morphology of *Convolvulaceae* with special reference to taxonomy. Review of Palaeobotanyand Palynolology13: 157–212.

Sheidai, M., Zanganeh, S., Haji-ramezanali, R., Nouroozi, M., Noormohammadi, Z. & Ghsemzadeh-baraki, S. 2013. Genetic diversity and population structure in four *cirsium* (*Asteraceae*) species. Biologia 68: 384−397.

Sheidai, M., Ziaee, S., Farahani, F., Talebi, S.Y., Noormohammadi, Z. & Hasheminejad-Ahangarani-Farahani, Y. 2014. Infra-specific genetic and morphological diversity in *Linum album* (*Linaceae*). Biologia 69: 32–39.

Soltis, D.E. & Soltis, P.S. 2000. Contributions of plant molecular systematics to studies of molecular evolution. Plant Molecular Biology42: 45–75.

Stamatakis, A. 2014. RAxML Ver. 8: a tool for phylogenetic analysis and post analysis of large phylogenies. Bioinformatics 30: 1312–1313.

Stefanovic, S., Kuzmina, M. & Costea, M. 2007. Delimitation of major lineages within *Cuscuta* subgenus *Grammica* (*Convolvulaceae*) using plastid and nuclear DNA sequences. American Journal of Botany 94: 568–589.

Stewart, G.R. & Press, M.C. 1990. The physiology and biochemistry of parasitic angiosperms. Annual Review of Plant Biology 41: 127–151.

Swofford, D.L. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods) Ver. 4.0b10. Sunderland: Sinauer Associates.

Weiss-schneeweiss, H., Tremetsberger, K., Schneeweiss, G.M., Parker, J.S. & Stuessy, T.F. 2008. Karyotype diversification and evolution in diploid and polyploid South American *Hypochaeris* (*Asteraceae*) inferred from rDNA localization and genetic fingerprint data. Annals of Botany 101: 909–918.

Welsh, M., Stefanović, S. & Costea, M. 2010. Pollen evolution and its taxonomic significance in *Cuscuta* (dodders, *Convolvulaceae*). Plant Systematics and Evolution 285: 83–101.

White, T. J, Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315–322. *In*: Innis, M., Gelfand, D., Sninsky, J. & White, T. (eds). PCR Protocols: A Guide to Methods and Applications. San Diego: Academic Press.

Wolfe, A.D. & Depamphilis, C.W. 1997. Alternate paths of evolution for the photosynthetic gene rbcL in four nonphotosynthetic species of *Orobanche*. Plant Molecular Biology 33: 965–977.

Young, N.D., Steiner, K.E. & Depamphilis, C.W. 1999. The evolution of parasitism in *Scrophulariaceae*/*Orobanchaceae*: Plastid gene sequences refute an evolutionary transition series. Annals of the Missouri Botanical Garden 86: 876–893.

Yuncker, T.G. 1932. The genus *Cuscuta*. Memoirs of the Torrey Botanical Club 18: 113–331

Yuncker, T.G. & Rechinger, K.H. 1969. Pp. 1–16. *In*: Flora Iranica, Vol. 8. Rechinger, K.H. (ed.). AkademischeDruck-u.-Verlagsanstalt, Graz.