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Full Length Article

Molecular Cloning and Sequence Analysis of the its Region of Nuclear Ribosomal DNA for Species Identification in Dodders (*Cuscuta*; Convolvulaceae)

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Abstract

Dodder (*Cuscuta* sp.) is an obligate parasitic plant that is very difficult to control. In plants the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal DNA (nrDNA) has been considered one of the most important sequences for phylogenetic analysis. Here we report the analysis of nrDNA's ITS sequences as an efficient tool to study the phylogeny of dodders collected from various provinces of Eastern Anatolia (Turkey). Genomic DNA of six dodder samples belonging to 4 distinct species was extracted from body tissue samples. The sequences of 18S rRNA, ITS-1, 5.8S rRNA, ITS-2 and 26S rRNA regions of 4 *Cuscuta* species were determined by molecular cloning and sequencing. The identity of cloned fragments was compared to determine sequence identity using NCBI database. Bootstrap analysis of nrDNA of *C. approximate*, *C. lupuliformis*, *C. campestris* and *C. babylonica* indicated high sequence identity with similar sequences belonging to different geographical origins of the world retrieved from NCBI database. Our results clearly showed that the most stable secondary structure derived from the sequences obtained by universal ITS4 and ITS5 primers is very efficient tool for identification of *Cuscuta* species when used in combination with phylogenetic analysis. © 2017 Friends Science Publishers

Keywords: Classification; *Cuscuta* spp; East anatolia; Identification; Molecular phylogeny

Introduction

The plant genus *Cuscuta* L. (dodders) is parasitic seed plants suppressing crop plants by competing with them for environmental resources that are needed for growth (Saeed and Zaroug, 1989). Members of the genus *Cuscuta* are stem parasites, mostly annual, pale stems, slender, herbaceous vines with twining with very little or no chlorophyll and no roots (Kujit, 1969; Cronquist, 1981; Stewart and Press, 1990; Garcia *et al*., 2014). Dodders are nearly cosmopolitan in distribution comprising some 200 currently described species (Yuncker, 1932; Hunziker, 1950; Mabberley, 1997; Stefanović *et al*., 2007; Garcia *et al*., 2014). These stem parasites are attached to the various hosts via haustoria and depend entirely on their hosts to supply nutrients and water (Kujit, 1969; Cronquist, 1981; Dawson *et al*., 1994).

There are numerous *Cuscuta* spp. found throughout the world causing serious problems on trees, herbaceous plants, forage legumes, shrubs and various crops (Li, 1987). *Cuscuta* is characterized by having scale-like leaves often with stomata, small terrestrial roots that quickly degenerate so that the mature plant is not connected to the ground and an embryo that is coil shaped without cotyledons (Rendle, 1959; Kujit, 1969; Searcy, 1970; Cronquist, 1981).

The Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA (nr DNA) has been widely used for identification and phylogenetic analysis of many angiosperm families. The complete ITS region is separated into ITS 1 and ITS 2. The ITS 1 is present between 18S and 5.8S rRNA whereas ITS 2 is present between 5.8 and 26s rRNA. 5.8S rRNA is a highly-conserved region (Baldwin *et al*., 1995).

In last two decades, numerous studies have been used the molecular data in seeking the relationships of flowering parasitic plants (Nickerent and Starr, 1994; Wolfe and dePamphilis, 1995; dePamphilis *et al*., 1997; Duff and Nickrent, 1997; Young *et al*., 1999; Stefanović and Olmstead, 2004). In cases in which morphological characters useful for phylogenetic inference are scarce the precise relationships of nonparasitic taxa remain uncertain. In these cases, nrDNA sequence data are often helpful to produce useful insights in biogeographical studies by using phylogenetic analyses (Yokoyama *et al*., 2000; Stefanović and Olmasted, 2004). In dodders, it has been demonstrated that the noncoding regions of chloroplast DNA can also be used to identify the species at a low taxonomic level (Stech and Frahm, 1999).

Today, the most widely used methods for the identification of *Cuscuta* species are based mostly on floral

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characters (Neyland, 2001) biogeographical knowledge, parasitic habit, vegetative characters associated with parasitism. Sometimes these characters are not sufficient for species identification in plants, due to low levels of variability. Even by comparative analysis of primary DNA sequences, there is a lack of consensus regarding the most technically practical, informative and universal DNA regions. Here we present a new molecular data set consisting of nuclear ribosomal DNA sequence and its most stable secondary structure, suitable to use for reliable identification of various *Cuscuta* species within Convolvulaceae. This approach involves two consecutive strategies: (i) molecular cloning and sequencing of nrDNA belonging to different species within the respective genera or families, (ii) bioinformatic analyses by constructing phylogenetic tree and the most stable secondary structures.

Materials and Methods

Collecting the Dodder Samples from Eastern Anatolia and Identification by Morphological Characters

The stem tissue samples of *Cuscuta* spp. were collected from different geographical regions (Erzurum, Malatya and Van provinces) of Eastern Anatolia (Turkey) as indicated in the Flora of Turkey (Davis, 1978) including agricultural and non-agricultural lands in July, 2015.

Herbarium specimens of *Cuscuta* spp. were deposited and description of each taxon was made based on inflorescence, flower, calyx, corolla, capsule, stigma, stylus and stamina bracts of examples and the seeds were dissected. A total of four molecularly studied species of *Cuscuta* samples from Eastern Anatolia and seventeen rDNA accessions including an out-group species were included in this study. Species names used in this study and their origin, length, AT and GC contents of ITS sequences, EMBL accession numbers, voucher information and sources are provided in Table 1.

Isolation of Genomic DNA and the Amplification of ITS Region

Stem tissues of the plants were studied both fresh and dried conditions. Genomic DNA from *Cuscuta* spp. was extracted by using a commercial DNA purification kit (Bioline, Luckenwalde, Germany). Identification was based on the flower structure, sequence analysis and secondary structure pattern of nrDNA.

DNA fragments of ITS region of nuclear ribosomal DNA (nrDNA) containing ITS1, 5.8S and ITS2 (here called ITS) were amplified from the genomic DNA by polymerase chain reaction (PCR) using universal primers (ITS4:5'-TCCTCCGCTTATTGATATGC-3'andITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*., 1990).

A final volume of 50 µL PCR mixture contained: 2 µL of DNA, 5 µL of 10×reaction buffer (200 mMTris–HCl pH: 8.4, 500 mMKCl), 3 µL of $MgCl_2$ (25 mM), 1 µL of dNTPs (10 mM each), 1 µL of each primer (100 pmol), 0.4 µL of Go Taq G2 Hot Start DNA polymerase, and 36.6 µL of DNase free sterile water. The following PCR program was set to denaturation at 94ºC for 2 min, followed by 36 cycles of 94ºC for 1 min, annealing at 55ºC for 1 min and an extension of 72ºC for 2 min, with a final extension of 72ºC for 10 min. The PCR product was separated on 1.5% agarose gel and recovered by gel extraction kit (Isolate II PCR and Gel Kit, Bioline, Germany). The DNA sequence of the final construct was verified using sequencing analysis. All the sequences studied in this study are deposited in GenBank database under the accession numbers given in Table 1.

Molecular Cloning and Sequencing

Amplified PCR products were purified with a commercial PCR Purification Kit (Bioline, Luckenwalde, Germany). The purified PCR fragments of ITS region was cloned into plasmid vector using TA Cloning Kit (Promega, USA) and transformed into competent *Escherichia coli* strain JM 109 by following manufacturer's instructions. The purified plasmids were sequenced by automated DNA sequencer (Applied Biosystems) at Iontek Research and Biotechnology Company. DNA sequencing was performed on both strands.

Secondary Structure Prediction

In order to predict the most stable secondary structure of the nuclear ribosomal DNA containing ITS1, 5.8S and ITS2 computer analysis was performed on the established full sequence after converting into RNA. For this purpose, themfold structure prediction package of CLC RNA Workbench Version 4.4 (CLC bio, Denmark) was used.

Sequence Retrieval and Phylogenetic Analyses

Phylogenetic analyses were conducted with nucleotide sequences retrieved online from the National Center for Biotechnology Information (NCBI)'s nucleotide sequence database (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi). Sequence similarity searches were performed using BLASTn function against the NCBI database. The phylogenetic tree was created using MEGA6 (Tamura *et al*., 2013) software under the maximum likelihood criteria. To root the phylogenetic tree, existing GenBank sequence of *Phytolacca americana* antiviral protein gene (Acces. No JX446580) was used as outgroup. The relationships were assessed using 1000 bootstrap replicates.

Results

Of the four *Cuscuta* species identified at Eastern Anatolian croplands, the all species were regularly found throughout the Eastern part of Turkey. None of the species identified by morphological characters exhibited morphological variation

Table 1: List of *Cuscuta* spp. used in this study and their origin, length, AT and GC contents of ITS sequences and EMBL accession numbers

Species	Other collection number	Origin	Source of nucleotide	Length (bp)	T(U)	C	A	G	G+C	A+T	EMBL accession no
C. approximata	--	Spain	NCBI database	611.0	27.0	23.6	218	27.7	51.3	48.8	DQ924598
C. approximata	1778	Van-Hosap/Turkey	This study	683.0	26.1	22.5	23.0	28.4	50.9	49.1	KU686677
C. approximata	12515	Erzurum	This study	682.0	26.0	22.6	23.2	28.3	50.9	49.2	KU725873
C. approximata	--	Pakistan	NCBI database	611.0	26.7	23.7	22.1	27.5	51.2	48.8	DQ924596
C. approximata	--	Greece	NCBI database	613.0	26.8	23.8	21.9	27.6	51.4	48.7	DQ924601
C. approximata	--	Pakistan	NCBI database	611.0	27.2	23.4	21.9	27.5	50.9	49.1	DQ924595
C. approximata	--	Canada	NCBI database	608.0	26.2	23.7	22.2	28.0	51.7	48.4	EF202561
C. lupuliformis	1784	Erzurum-Cat/Turkey	This study	680.0	22.1	26.9	22.1	29.0	55.9	44.2	KU707914
C. lupuliformis	--	USA	NCBI database	577.0	22.0	28.1	19.9	30.0	58.1	41.9	EU330321
C. lupuliformis	--	UK	NCBI database	741.0	22.1	27.4	21.5	29.0	56.4	43.6	AY554404
C. lupuliformis	--	Germany	NCBI database	609.0	23.2	27.9	21.5	27.4	55.3	44.7	DQ924570
C. lupuliformis	--	China	NCBI database	410.0	22.4	27.3	20.5	29.8	57.1	42.9	KF454368
C. campestris	1764	Van-Baskale/Turkey	This study	670.0	27.2	22.2	22.5	28.1	50.3	49.7	KU725869
C. campestris	--	Canada	NCBI database	640.0	27.8	22.8	21.7	27.7	50.5	49.5	KT383151
C. campestris	--	Canada	NCBI database	661.0	27.8	22.4	22.4	27.4	49.8	50.2	KT383102
C. campestris	--	USA	NCBI database	648.0	27.8	23.0	22.1	27.2	50.2	49.9	KT383163
C. campestris	--	South Africa	NCBI database	611.0	28.0	22.9	21.6	27.5	50.4	49.6	KT383188
C. campestris	--	Unknown origin	NCBI database	661.0	27.5	22.5	22.4	27.5	50.0	49.9	KT383149
C. babylonica	1820	Van-Ozalp/Turkey	This study	669.0	29.6	20.2	24.8	25.4	45.6	54.4	KU725870
C. babylonica	1780	Malatya/Turkey	This study	668.0	29.6	20.4	24.4	25.6	46.0	54.0	KU761258
C. babylonica	--	Afghanistan	NCBI database	596.0	30.4	21.5	23.5	24.7	46.2	53.9	DQ924579
C. babylonica	--	Turkey	NCBI database	596.0	30.0	21.6	23.3	25.0	46.6	53.3	DQ924578
Phytolacca americana antiviral protein gene	--	Outgroup	NCBI database	--	--	--	--	--	--	--	JX446580

throughout its range and no sequence divergence was found between the same species samples from Van and Erzurum provinces.

The species belonging to genus *Cuscuta* that were previously reported in Eastern Anatolia were identified by morphological and molecular basis. Species identification in the genus *Cuscuta* is sometimes difficult and requires the use of morphological and biogeographical knowledge and the host range of the species.

The procedures we describe here, when used in combination, provide a rapid tool for identification of parasitic dodders. Performed molecular assays showed that the partial sequence data of ITS region containing 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence are enough for the identification and classification of *Cuscuta* species studied in this paper. ITS sequences, including the 5.8S gene, were determined for 4 *Cuscuta* species from 6 samples and their EMBL accession numbers for these sequences and further details of sequence characteristics are provided in Table 1.

The entire ITS region (ITS1-5.8S-ITS2) has been generated successfully using the reported ITS4 and ITS5 primers and templates of total cellular DNA of *Cuscuta* spp. As shown in Fig 1.

Six *Cuscuta* samples studied in this research were differentiated into four main species by ITS sequences, which were designated as I, II, III and IV groups. The sequences of *C. campestris* were grouped in Group I, likewise the sequences of *C. lupuliformis*, *C. approximata*, and *C. babylonica* were grouped in Group II, Group III, and Group IV, respectively. In the tribe Fabaceae (Chennaoui *et al.*, 2007) and in many other genera in angiosperms (Baldwin *et al*., 1995) similar results were reported for the same region of ITS. Bootstrap analysis indicated a little divergence among *Cuscuta* species. The resulting tree obtained by MEGA6 was similar in topology to that of the consensus parsimony tree obtained by BLASTn function of NCBI's nucleotide sequence database. Genotypes of *C. approximate*, *C. lupuliformis*, *C. campestris* and *C. babylonica* were separated as distinct clusters by maximum likelihood and maximum parsimony tests. Four well-supported clades (I, II, III and IV) are resolved among compared *Cuscuta* ITS sequences.

Based on BLAST report, *C. babylonica* (sample no

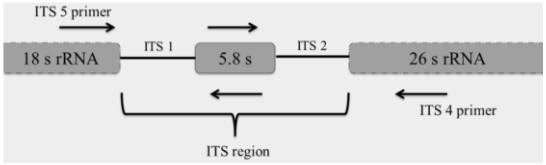

Fig. 1: Schematic representation of nuclear ribosomal DNA (nrDNA) contains ITS1, 5.8S and ITS2 with primer binding locations

1780) from Malatya were exhibited high sequence identity with 0.0 e-value (Fig. 2). The species clearly clustered with the individual from same species.

The topology of the assembled secondary structure model of two samples of *C. approximata* and two samples of *C. babylonica* from Eastern Anatolia were similar in these analyses because they were identical at all potentially molecular informative sites (Fig. 3a–d).

Discussion

The morphological differentiations of the major species of *Cuscuta* do not necessarily represent genetic differences among the dodder species. Identification of species by classical methods is time consuming task and always requires expertise.

Previous classifications of *Cuscuta* mainly relied entirely on flower and fruit characteristics (Yuncker, 1932) and phytochemicals (ul Hassan *et al.*, 2014). Later systematic investigations, however, revealed that the majority of morphological characters in *Cuscuta* are significantly affected by convergent evolution. This was resulted some unavoidable morphological overlapping. Therefore, the predictive value of new molecular characters is high, in addition to morphological and biogeographical characters (Costea *et al.*, 2015). The present molecular investigations on *Cuscuta* species identity have yielded significant knowledge about species identification. In the present study, the nuclear ribosomal DNA internal transcribed spacer region (ITS) of 4 *Cusuta* species were cloned and sequenced. DNA sequencing revealed little genetic variation of *Cuscuta* species compared with the same species in databases.

Here, we present a combination of two methods to increase the efficacy of diagnostic screenings *Cuscuta* species. To achieve this goal, first nrDNA have been cloned and sequenced than phylogenetic tree and the most stable secondary structure were constracted. At the first step, phylogenetic position of the nrDNA of a given species was investigated. The all species, studied in this research, were clustered with the similar sequences of the same species. At the second step, the most stable secondary structures were found almost identical for same species (Fig. 3a, b, c and d) supporting the detection. Capability of our approach, consisting of the combination of two molecular analyses, was found applicable and successful in detecting and identifying dodder individuals at species level. Combination of the two bioinformatic methods can be considered a cheap and simple means for the molecular identification of Cuscuta species.

Our study based on secondary structure and sequence analysis of nRDNA supports distinct taxonomic categories of *C. approximata*, *C. lupuliformis*, *C. campestris*, and *C. babylonica* species; however, further efforts are highly needed to measure similarity and dissimilarity in the secondary structure of a reference and a given sequence

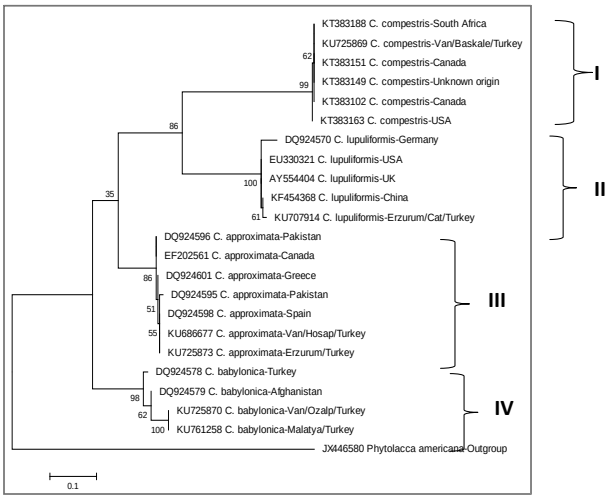

Fig. 2: Phylogenetic analysis of the six ITS sequences observed among the species of *C. approximata*, *C. lupuliformis*, *C. campestris* and *C. babylonica*. The heuristic search was obtained by applying under maximum likelihood and maximum parsimony criteria. Numbers on the branches represent the 1000 bootstrap replications in which a given branch appears

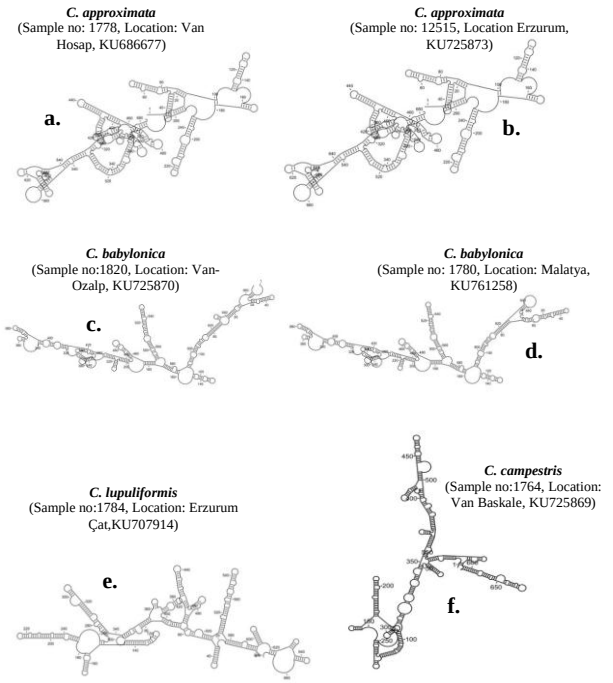

Fig. 3: Secondary structural models of nrRNA of *Cuscuta* samples studied in this paper. Note the similarity of structurally conserved stem and loop regions of *C. approximata* (a and b) and *C. babylonica* (c and d) and dissimilarity of *C. lupuliformis* (e) and *C. campestris* (f)

based on their respective base pairing probability matrices to clarify the taxonomic assignments at species and variety level for other members. Therefore, the applicability of method

to other species and varieties may also be investigated.

Schoch *et al.* (2012); reported that the number of variable nucleotide positions identified in ITS sequences depends on the way of sequencing. In our case, especially if determined after cloning, ITS was found a powerful tool to distinguish closely related *Cuscuta* species. Our approach to use RNA secondary structure prediction at identifying *Cuscuta* spp. and the classification of species has proven effective. Advantage of this approach is that it can be used by any software able to predict the most stabile secondary structure of a given RNA molecule. The pattern of secondary structure is compared to that of similar pattern of the same species. The main drawback of this approach can be considered the requirement to specific nucleotide sequences of rRNA of a given species prior the application of the test.

Conclusion

Based on our findings, it is concluded that the distinctive structural and molecular patterns of noncoding nrDNAs are a prerequisite for the proper diagnosis of many *Cuscuta* species. The ITS region of nuclear ribosomal DNA of *Cuscuta* spp. seems to be long enough to provide sufficient data for identifying and classifying the species. The combined molecular methods described here will provide powerful tool for the identification of economically relevant species within this plant parasitic genus.

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