Identification of Cuscuta campestris Yuncker in UAE: study of bar code loci- rbcL, matK and trnH-psbA in the UAE and Egyptian cultivars and in the respective host plants basil and jute

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Abstract*— Cuscuta campestris is a stem holoparasite. We observed Cuscuta parasite on basil host plant Ocimum basilicum, in Al Mohadub Umm Al Quwain, UAE. The parasite was pale green in color, twined around the host in anti-clock wise direction, with white flowers that had green ovaries at maturity. Based on the morphology and floral structures, we identified the parasite as C. campestris Yuncker. To authenticate the species, three "Bar-code loci" viz, rbcL, matK and inter-spacer region trnH-psbA were studied. A portion of rbcL locus and the trnH-psbA non-coding spacer region seem to be intact, revealed by PCR amplification and sequencing, while three sets of primers failed to amplify the maturase K locus. Although the stem and floral structures were light green in color, RuBisCo protein could not be detected in polyacrylamide gels, indicating its total dependency on the host at that stage of development. To validate thus obtained results, frozen samples of C. campestris were collected from Egypt and the three bar code loci (rbcL, matK and trnH-psbA) were amplified with the same set of primers; the PCR products were sequenced. There was 100% similarity with respect to the sequenced loci (rbcl and trnH-psbA) between the two cultivars of C. campestris Yuncker. Sequences were deposited in Genbank with accession numbers KXO15762 (C. campestris, UAE) and KXO15761 (C. campestris, Egypt). C. campestris is being reported for the first time from Al Mohadub Umm Al Quwain, UAE. There is no difference in both the candidate bar code gene loci rbcL and trn-H psbA between the UAE and Egyptian cultivars of Cuscuta campestris and the region is conserved.

Key words: Cuscuta, campestris, rbcL, matK and trnH-psbA.

INTRODUCTION

Parasitic plants are a unique category in plant kingdom that depends on other plants completely or partially for their survival. *Cuscuta commonly known as dodder,* has about 200 species which are either holoparasites or hemiparasites (CABI, 2016). The genus is easily identified through its morphology of thin, pale green, leafless twining stems that bear flowers. However, species of *Cuscuta* are identified based upon the shape and size of calyx and corolla, morphology of style and stigma and capsule dehiscence (see McNeal, 2007). However, the small size of flowers and the inconspicuous floral structures throw challenge to taxonomists for correct identification of the species. Correct identification of the parasite is mandatory as different species of *Cuscuta* cause gross losses in the crop yields that range from 86-18% for different crops that they infect (Mishra et al., 2007). Besides, dodders are difficult to eradicate as they are known for easy spread through their seed which survive for many years in soil. Early detection and correct identification of the species is a prerequisite for its eradication of the parasite which is attempted in the present study.

During the last decade, use of plant bar code has gained momentum in identifying the plant species; more than one gene has been suggested as candidate bar code loci for appropriate identification of the species. Consortium for the bar code of life plant working group (CBOL, (2009) suggested RbcL and matK as bar code loci, while trnH-psbA was suggested by Kress and Erickson, (2007). It was opinioned that this single non-coding locus trnH-psbA will discriminate all the angiosperm species with a one primer pair (Shaw et al., 2005 and 2007). In the present study rbcL, matK and trnH-psbA loci have been studied for accurate identification of *Cuscuta* species. Recently in 2011, China Plant BOL group suggested nuclear transcribed spacer ITS2 as a candidate bar code locus that would have better discriminatory power than plastid bar codes (rbcL and matK) in species identification, which too is included in our study (Li et al., 2015). With an aim to study the effect of environment, samples of *C. campestris* were collected from Egypt which are included in the present study.

Conflicting data exist on the photosynthetic ability across the genus – *C. reflexa*, *C. pentagona* and *C. campestris* had normal ratio of chlorophyll a to chlorophyll b (Nickrent et al., 1997), low levels of chlorophyll in *C. reflexa*, restricted occurrence of chlorophyll in *C. gronovii* (Funk et al., 2007), while there was no detectable chlorophyll in some species like *C. europaea* (Delavault et al., 1 996). In the present study RuBisCO protein also was estimated in the chlorophyllous stems and flowers of *Cuscuta* with an aim for the correct identification of the species.

MATERIALS AND METHODS

Morphological study: The number of floral organs, sections of flower was carried to identify the species.

DNA extraction: Genomic DNA was extracted from ≈400mg of leaves (host) or stems of parasite, using Norgen fungal/plant DNA isolation kit, following the manufacturer's instructions. The extracted DNA samples were electrophoresed on 1% agarose gel at 100 V, the gels were then viewed and photographed under UV illumination to estimate the concentration of the DNA µg/µl.

PCR assays: Primers and 2 X PCR master mixtures were purchased from Norgen, Canada. A 20 µl PCR reaction was performed which contained 2 µl (≈50 ng) of extracted genomic DNA, 10 µl of 2X PCR master mix, 1 µl (10 µM) of each primer forward and/or reverse, 6 µl of sterile distilled water using a thermal cycler (Veriti PCR Machine, Applied Bio systems, USA). The PCR amplification conditions were 94ºC for 3 min, followed by 1 min at 94ºC and 72ºC for 1min for 30 or 35 cycles with a final extension at 72ºC for 10 min. The annealing temperature was different for different primer sets. The PCR products were electrophoresed on 1.0% agarose gel at 100 V, the gels were viewed and photographed under UV illumination.

Table 1: Primer sequences used in the present study.

Gene	Primer	Sequence of primer
ITS	ITS-S2F (F)	5'- ATG CGA TAC TTG GTG TGA AT -3'
	ITS4 (R)	5'- TCC TCC GCT TAT TGA TAT GC -3'
Rbc L	rbcLa-F (F)	5'- ATG TCA CCA CAA ACA GAG ACT AAA GC -3'
	rbcLa-R (R)	5'- GTA AAA TCA AGT CCA CCR CG -3'
mat K	matK-KIM3F	5'- CGT ACA GTA CTT TTG TGT TTA CGA G -3'
	matK-KIM1R	5'- ACC CAG TCC ATC TGG AAA TCT TGG TTC -3'
	matKpkF4	5'- CCC TAT TCT ATT CAY CCN GA -3'
	matKpkR1	5'- CGT ATC GTG CTT TTR TGY TT -3'
	matK-390f (F)	5'- CGA TCT ATT CAT TCA ATA TTT C -3'
	matK1326 (R)	5'- TCT AGC ACA CGA AAG TCG AAG T -3'
psbA -trnH	psbAF (F)	5'- GTT ATG CAT GAA CGT AAT GCT C -3'
	trnH2 (R)	5'- CGC GCA TGG TGG ATT CAC AAT CC -3'

Extraction of RuBisCO protein: Extraction of RuBisCO protein from the stem and flowers of *Cuscuta campestris* and leaves of basil plant: About 500 mg of the tissue was weighed and ground in mortar and pestle with liquid nitrogen into fine powder. The protocol was followed as suggested by Simpson (2006). 2 ml of the maceration buffer was added which contain (200 mM Tris-Cl, 10 mM EDTA, 1 mM DTT, 0.05% tween 20, 10% glycerol) and incubated for 5 min. The homogenate is centrifuged at 12,000 xg for 20m in a refrigerated centrifuge. The 20 µls supernatant is mixed with 10 µls of Laemmli 2X buffer (4% SDS, 10% 2-mercaptoehtanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl and pH adjusted 6.8). The proteins were denatured by boiling at 90^0 C for 10 min and were loaded on 15% SDS PAGE. The gel was electrophoresed for 3 hrs first one hour at 100 volts and later at 150 volts .The gel was stained with instant Coomassie stain for one hour and was destained in 30:10 methanol and acetic acid solution. Proteins were extracted from stems and flowers of *Cuscuta campestris*, basil leaves, and spinach leaves.

Sequencing: The purified PCR products were sequenced by Macrogen, Korea. The DNA nucleotide sequences are blasted in NCBI search and identified.

RESULTS AND DISCUSSION

Morphology: The stem of *Cuscuta* were pale green, branched without leaves; bore pedicillate flowers in 2 or 3 in compact clusters at each node. Each flower had 5 sepals, 5 petals, 5 stamens and usually 3 carpels. Calyx lobes are ovate, corolla lobes fleshy and pointed; perianth is white and cup shaped. Stamens are 5, filaments with basal attachment to the anther lobes, alternate with the corolla lobes (Fig. 1A). Ovary is white (Fig.1B) or green in color at maturity, (Fig.1A); each has 2 styles (Fig.1A, blue arrow), each style is with capitate stigma (Fig.1A, white arrow). The ovary in transverse section showed 3 carpels, each carpel contained ovules on axillary placentation. Seeds were oval or pear shaped or irregular and grey in color (Fig. 1C), abundant in the capsule; not readily visible as the perianth persisted on the capsule at maturity. From the morphological study with reference to Bolus (2000), we identified the parasite as *C. campestris*. *C. campestris* is often confused with *C. pentagona* which is characterized by angular Calyx (McNeal, 2007), but the present species lacks the morphological marker, hence is identified as *C. campestris.* Three more species- *C. gronovii, C. australis* and *C. chinensis* resemble *C. campestris* with similar morphology and often are mis-identified as *C. campestris* (Parker and Riches, 1993).



Figure 1: **1A:** Flowers and seeds of *C. campestris*, **1B:** Flowers of *C. campestris* with green carpels, **1C:** seeds of *C. campestris.*

C. australis has rounded corolla lobes, *C. chinensis* has fleshy corolla lobes and does not set seed easily (McNeal, 2008) and *C. gronovii* is not reported outside USA so far (Parker, 2008) implying that the present species could be *C. campestris* which could be authenticated by matching the amplified barcode sequences with the deposited sequences in genbank NCBI.

DNA extraction: DNA could be extracted from the two parasites of *C. campestris*, and the respective host plants basil and jute. The yield of the DNA ranged between 500 - 800 µg/µl among the four plants.

PCR amplification and sequencing: For each set of primers different annealing temperatures were tried and standardized before repeatable results were obtained, the most suitable annealing temperature that enabled PCR amplification

is shown in Table 2. The gene segments of ITS, rbcL and spacer region of trnH-psbA are successfully amplified with the respective sets of primers among the two parasites and the two host plants (Fig.2). Amplification of the isolated genomic DNA indicated the quality of the DNA to be good which a pre-requisite for consistent PCR amplifications.

Amplification with ITS-S2F and ITS4 primers confirmed that it is plant genomic DNA and not fungal DNA (Fig. 2A). The amplified 700bps fragment of RBCL gene was eluted and sent to Macrogen, (Korea) for sequencing. A set of primers of matK gene -matK-390f and matK132r amplified the host DNA (basil and jute), with a 900 bps amplification product (Fig. 2 E), three sets primers failed to amplify in either cultivar of *C. campestris*, which could be due to gene loss and/ or failed amplification (Fig.2D); *Cuscuta* species lose many genes (McNeal, 2007) to have compact DNA (Funk et al., 2007). Although the plastid DNA has been sequenced \in the present study, there is a possibility that this locus matK must be impaired.

Interestingly, the primers of trnH-psbA revealed **"multiple bands"** in the host plant of 450bps and 250bps, while they were of~ 250 bps in each of the parasite plants (Fig.2C).The multiple bands in the host plants indicate multiple annealing sites for the each primer. Multiple bands with this non- coding spacer region between trnH and psbA are expected based on which the sequence was suggested as a mandatory bar-coding sequence to decipher the varietal differences (Kress et al, 2005).

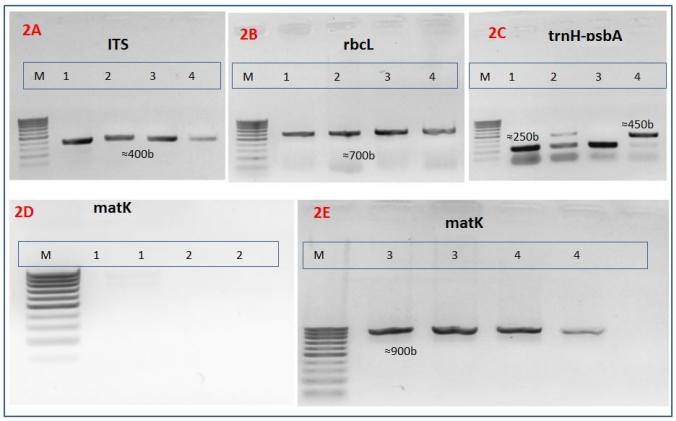

Figure 2: PCR amplifications of ITS, rbcL, trnH-psbA and matK gene loci. M= Marker, 1= *Cuscuta campestris*, Egypt, 2= *Cuscuta campestris,* UAE, 3= *Ocimum basilicum* and 4= *Corchorus capsularis.*

Table 2: annealing temperature for each set of primers and the PCR products.

Gene	Primer	Annealing temperature no of cycles	PCR product size
ITS	ITS-S2F (F)	**54°C** 1m, 30	≈400bps
	ITS4 (R)		
rbcL	rbcLa-F (F)	**55°C** 1m, 35	≈700bps
	rbcLa-R (R)		
matK	matK-KIM3F	-	
	matK-KIM1R	-	
	matKpkF4	-	
	matKpkR1	-	
	matK-390f (F)	**51°C** 1m, 30	≈900bps
	matK1326 (R)		
psbA -trnH	psbAF (F)	**56°C** 1m, 30	250-600 bps
	trnH2 (R)		

However, the present results do not uncover any differences between the two parasites collected from the two ecological niches (results shown later), or even two different genera- *Ocimum* and *Corchorus*, if the agarose gel images are dependable.

Sequencing: The nucleotide sequences PCR of rbcL was blasted in NCBI and BOLD search and identified based on sequence similarity. The sequence matched 100% with 96% query and a score 562 of 569 confirming that it is *C. campestris*. Sequence alignments of the two sequences, rbcL and trnH-psbA of the two cultivars of *C. campestris* collected from UAE and from Egypt did not reveal any differences except for 2 nucleotides in rbcL (Fig.3a) or at the terminus indicating PCR errors (Fig.3b), the sequences are deposited in NCBI Genbank for which accession numbers KXO15762 (*C. campestris*, UAE) and KXO15761 (*C. campestris*, Egypt) are obtained.

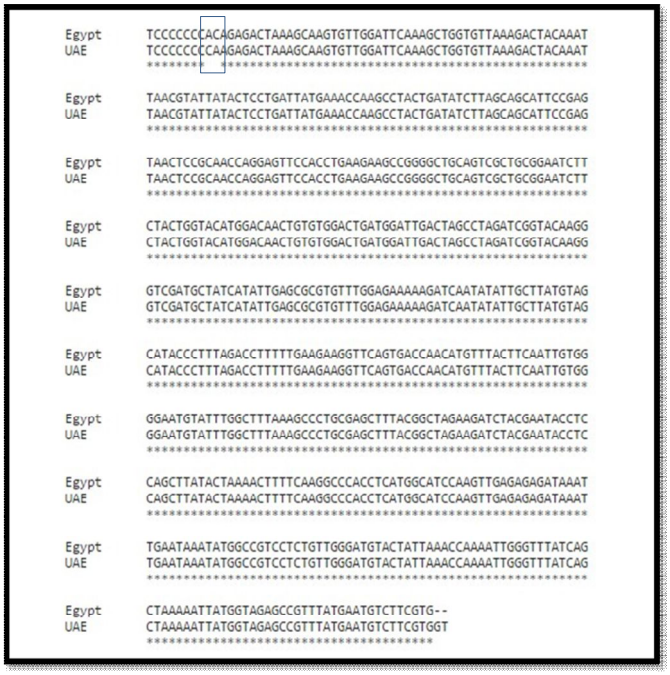

Figure 3a: Alignment of rbcL sequence of *C. campestris* from UAE and Egypt.



Figure 3b: Alignment of trnH- psbA sequence of *C. campestris* from UAE and Egypt.

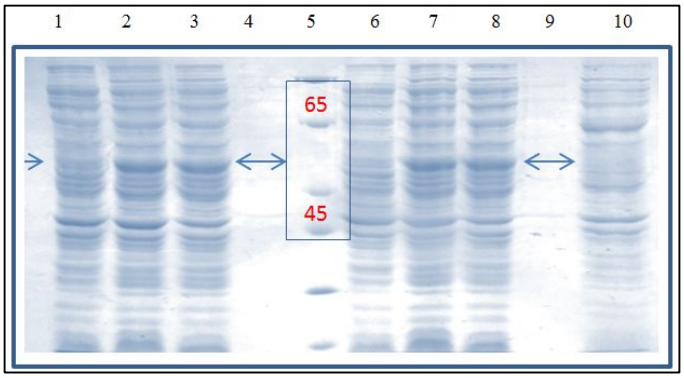

Figure 3b: 15% SDS PAGE showing RuBisCO protein. 1= *C. campestris* stem Egypt, 2= Jute leaves, 3= spinach leaves
4= *C. campestris* flowers, 5= Fermentas Protein Marker.
6= *C. campestris* stem UAE, 7= basil leaves, 8= Spinach leaves
9= flowers, absence of Rubisco protein, 10= stems of *C. campestris* UAE.

protein of 55 Kdsa could be detected in the basıl leaves, spinach but could not in the stems or flowers of parasite. RuBisCO protein is either not present or in minute quantity in *Cuscuta campestris* (Fig.4). It is reported that *C. campestris* has very low number chloroplasts with 1-2% of photosynthesis compared to normal autotrophs (CABI, 2016). However, the present study does not confirm presence or absence of RuBisCO; Western hybridization will enable us to detect the presence of small quantities of RuBisCO protein if any, which is underway.

CONCLUSION

The present parasite noticed in Al Mohadub Umm Al Quwain is identified as *C. campestris i*s being reported from UAE for the first time. The coding sequence rbcL and the non-coding spacer region trnH-psbA seem to be conserved in the UAE and Egyptian cultivars of *C. campestris* which are grown in different ecological niches.

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AUTHORS' CONTRIBUTIONS

Author 1 learnt the techniques from 4, standardized all the experiments, demonstrated to authors 2 and 3, analyzed sequencing data. Authors 5&6 drafted the MS, 7 designed the experiment, analyzed the data and wrote the MS.

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