The contraction in inverted repeat regions in the complete plastome sequence of *Cressa cretica* L

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Abstract

Plastome studies have been the focus of research in plant molecular evolution and systematics. *C. cretica* L. (Convolvulaceae) is a halophyte, habitat in the ecologically challenged area with high salinity and drought. The complete physical map of plastome revealed that it is 141,419bp long, circular molecule. It contains typical quadripartite structure of large single copy region (LSC 94,808bp), small single copy region (SSC 32,527bp) separated by a pair of inverted repeat regions (IRs 7042bp). This plastome is compared with the complete plastomes of other members of Convolvulaceae showed notable distinctions. An exceptional shift in IRs to SC regions is experienced in *C. cretica* led to many genes shift in both SC regions and contraction in IRs. The size of IRs reduced to 2 to 4 times as compared to those of the Convolvulaceae members studied. The shifted IRs regions showed remarkable variation in nucleotides patterns. Further, the shift was from the IR boundaries and in between the IR regions led to segment IRs. It is concluded that the shift in IRs may be the strategic move for adaptation in the harsh environment.

Key words: *C. cretica*, contraction in genome, inverted repeat regions, plastome

Introduction

The higher plant cells, three compartments contain their own DNA of which the chloroplast harbors the least complex genetic material. The chloroplast genome (plastome) exists as a covalently closed, double stranded circle ranging in size from 85-217 kb, but most common land plants contain 115-164kb. With few exceptions, higher plant chloroplast DNA contains two inverted, exact repeats of 20 to 30 kb which are separated by a small and a large single copy region. Thus a typical circular plastome has a quadripartite structure and exhibits highly conserved gene order and contents (Jansen et al. 2005; Wicke et al. 2011). The number of genes ranged from 63-209 among various lineages, a common pattern of most plastomes showed average 110-130 which includes protein coding, rRNA, and tRNA genes. However, large-scale genome rearrangement and gene loss have been identified in several angiosperm lineages (Wolfe et al. 1992; Lee et al. 2007).

The key important feature of chloroplast DNA is the presence of two large inverted repeats which reduced the rate of new recombination, mutations and promote DNA repair mechanism hence slow down the rate of evolution. Dynamic changes in IRs as expansion/contraction play a decisive role in plastome evolution. In number of studies such as Apioideae (Plunkett and Downie 2000; Downie and Jansen 2015), monocots (Wang et al. 2008), ferns (Wolf et al. 2010) and Pinaceae (Lin et al. 2010) reported that IRs contributes in increase /decrease in size of plastomes and gene rearrangement (Magee et al. 2010).

Investigation of genome size changes in various organisms found correlations with cytological, physiological, and ecological characters (Jockusch 1997; Gregory 2002; Knight and Ackerly 2002), even within single species (Bennett and Leitch 1995; Nevo 2001). *Cressa cretica* (Convolvulaceae) is a perennial halophyte which dominates both inland and coastal marshes. *C. cretica* seeds can germinate in up to 850mM NaCl and it can tolerate up to 950mM NaCl which is one of the highest concentrations (Priyashree et al. 2010). However, little is known about a molecular aspect of this plant. In this study, it is collected from desert of Little Runn of Kutch (Western India). Kutch desert is a unique ecosystem, with an admixture of saline, marshy and coastal desert where water and soil are extremely saline. It also demonstrates high temperature (~48˚ C) in hot summer and as low as 10˚ C in winter cold waves. Rainfall is

also not adequate (~320 mm). In this harsh environment, this area harbors it own exceptional flora, with some endemic and the species of high conservation significance at national and international levels.

In this paper, we investigate the organization and evolution of cp genome of *Cressa cretica* and it is compared with the available complete plastomes of other members in the Convolvulaceae in the GenBank. The unusual features of IR contraction in the genomes of *C. cretica* are described which provide valuable insights into cpDNA evolution. The comparisons with other members of Convolvulaceae identify multiple inversions, gene duplications, IR contraction, gene and intron losses. Gene relocation caused by IR loss in SC region is a notable feature of the cp genomes of the *Cressa cretica.*

Results and Discussion

Genome content and organization

The size of the plastome of *C. cretica* is 141,419bp with typical quadripartite structure, including a LSC region of 94,808bp and SSC region of 32527bp separated by a pair of identical IRs of 7042bp each (Fig.1). The total plastome size is consistent with those from other angiosperms which range from 85 to 176 kb. The complete plastome of *C. cretica* is slightly shorter than that of *Ipomoea* species (161-162 kb) but larger than those of *Cuscuta* species (85-125 kb) which all belong to the Convolvulacease (Table-1). The length of LSC region in *C. cretica* was slightly higher to *Ipomoea* species but much larger than that of *Cuscuta gronovii* and *C. obtusiflora.* In *C. exaltata* and *C.reflexa*, LSC region recorded lower values than that of *C. cretica* and *Ipomoea* species (Table-1). In contrast to other members of Convolvulacease, *C. cretica* showed extended SSC region from both ends of IRs, the largest size i.e.32.527 kb (Table-1, Fig.1). Further, in *C. cretica*, a total number of the protein coding genes were 82, total 27 tRNA coding genes for 21 different tRNAs, and 8 rRNA genes for 4 rRNA were observed. It has near to equivalent numbers of protein coding, tRNA and rRNA genes to *Ipomoea* species (Table-2).

GC content

Plastome GC content is highly conserved in land plants and is typically in the range of 30-40%, with GC content being lower in non-coding regions than in coding regions (Bock 2007). In Convolvulaceae plastomes, an overall higher range of GC content was observed 37.5-38.6% (Table-2). In all the members of Convolvulaceae, higher GC content observed in rRNA and tRNA genes (50-55%) than in protein coding genes (37.5-39%). The overall G+C content was slightly higher in *C.cretica* than other members of Convolvulaceae.

IR loss

The outstanding structural feature of plastome of land plants studied so far is a pair of large inverted identical repeat (IRs) sequence, which varying in length from 15-30kb in different species (Raubeson and Jansen 2005; Bock 2007). In Convolvulacease family, considerable length variation exists between the plastomes; with the smallest IR (7042bp) is observed in *C. cretica* less than one-forth size of that in *Ipomoea purpurea* and *I.nil,* less than two and half size of *I. batatas* while nearly less than half size of IR in *Cuscuta* species (Table-1). Slight variability in the size of land plan IRs is due to expansion and contraction of IR boundaries into single copy regions (Raubeson and Jansen 2005; Bock 2007); however, the extent of IR contraction in *C. cretica* is unprecedented. Here, the region is more than 2.5 to 5 times smaller than the typical land plants IR including the members of Convolvulacease compared (Table-1).

Notably, IR regions divided into four segments; IRaI to IRaIV and IRbI to IRbIV (Fig.2a). The loss of base pairs in IRa and IRb also observed significantly different. Between IRaI to IRaIV, the loss observed for 17, 271 and 3449 bp, respectively; while in IRbI-IRbIV, it was 37,270 and 3737bp, respectively. Thus a total loss of 3737 (in IRa) and 3796 (in IRb) was observed, with a difference of 59bp loss at IRa site (Fig.2a). In LSC, IR boundary shift was evident with *trnL-CAA* and *ycf2* (Fig.2b). Further, the genes shifted in SSC from IRa and IRb also aligned showed a significant difference in intergenic as well as in coding regions of *rps7, rps12, ycf15* and *ndhH* genes (Fig.2c). Pairwise alignment of nucleotide sequences of IRa and IRb regions, shifted in either LSC or SSC regions, showed significant nucleotide variations (Table-3). More polymorphic sites were observed in LSC (225) than in that of SSC (16) and indel sites were also

higher in LSC (812) than in SSC (44). These data suggest that the loss of IR region is more towards the LSC region. LSC/IR and SSC/IR boundaries are sometimes regarded as an index of chloroplast evolution. As observed in other plastome studies, the IR contraction has led to changes in the structure of the chloroplast genome, contributing to the formation of pseudogenes (Saski et al. 2005; Zhang et al. 2013; Luo et al. 2014). In this study, we have observed *rps7* and *ycf2* on IRb shift and *ndhH* on IRa shift as pseudogenes.

Gene rearrangement

IR contains a core rRNA and tRNA cluster, this includes four rRNA genes for 4.5S,5S,16S and 23Sand five tRNA genes encoding trnA-U-GAC, trnI_GAU, trnN-GUU, trnr-ACG and trnV-GAC (Jansen et al. 2005). In addition, IR region also contains a variety of other genes as a result of lineage-specific expansion and contractions (Zhu et al. 2016). However, in *C. cretica*, each IR includes only 2 copies of *trnl-GAU,* two copies of *trnA-UGC, orf56, orf42, rrn16, rrn23, ndhB* and *ycf68*: remaining IR region shifted in SC region. In SSC, genes for *rps7, rps12, ycf15, trnV_GAC, rrn4.5, rrn5, trnR-ACG, trnN-GUU, ycf1* and *ndhH* were observed in two copies, however, significant substitutions among the copy of these genes were observed. One copy of *rps7* and *ndhH* observed as pseudogenes (Fig.S1). In general SSC region contains the single copy of NADH oxidoreductase genes *ndhA,ndhD, ndhE, ndhF, ndhG, and ndhI* along with genes for photosystem I- *psaC,* ribosomal protein small subunit *rps7, rps15* and ribosomal protein large subunit and *rpl32* On the other hand, in LSC shifted region of IRs *trnL-CAA* and *ycf2* were observed in two copies with remarkable nucleotide variations (Table-3, Fig.2b). The shifted regions showed nucleotide variations in either gene sequences (*rps7, ndhH and ycf2*) or the shift was due to changes in intergenic regions (Fig.2&4 Table-4). *ycf2* found reduced in size like pseudo alternative of *ycf2* found in *Cuscuta reflexa*. Other than highly diverse *ycf2* one copy is pseudo in *Cressa*. In *Cressa ycf2* shifted in SSC is 2502bp in size while of LSC (pseudo) is 2498bp in size. *ycf2* of other convolvulaceae members are 6606bp of *Ipomoea nill,* 6627bp of *I.batata*, 6594bp of *I. purpurea,* 6717bp of *Cuscuta exaltata,* 5415bp of *C.gronovii,,* 5394bp of *C.obtusiflora* and 6012bp of *C.reflexa* (Fig. 3). In all three shifted genes from IR of *Cressa* shows strong node support for phylogenetic relationship (Fig.S1) by giving 100% bootstrap value while poor bootstrap observed for *Ipomoea nill*. To further confirm

evolutionary pressure for these three shifted genes Ka/Ks ratio was calculated and values obtained are 3.56 for ycf2, 3.62 for rps7 and 3.41 for ndhH. Thus >1 value of Ka/Ks confirms mutation biases among these genes and shows positive selection pressure (Yang 1998; Chen et al. 2017) on these protein coding genes (S Table-S1).

The existence of IRs may confer on the plastome by a formation of head-to-head dimmers. IRs represents hotspots for resistance to intra molecular recombination loss and support stabilization of plastome against rearrangement (Wicke et al. 2011). Nevertheless, these potential functional implications do not prevent the variation of IRs. On the other hand, IRs is frequently subjected to expansion, contraction or even complete loss (Palmer et al. 1987; Tsudzuki et al. 1992; Guo et al. 2014). The IR region of other Convolvulaceae plastomes was highly conserved but structure variation still found in IR/SC boundaries (Fig.3). To elucidate the potential contraction of IR regions, we compared the gene variation at the IR/SSC and IR/LSC boundaries regions of eight plastomes. The gene trnH-GUG was observed in all Ipomoea species and *Cuscuta* species including *N. tobacco* as an outgroup, at the junction of LSC/IRb region whereas in *C. cretica* at the LSC/IRb boundary trnI-CAA was observed followed by 468bp extension touching to the IRb boundary. In IRb region, an initial 29bp region was observed before *ndhB*. Two copies of *ndhB* crossed IRb and SSC with an intron sequence in *C.cretica*. Although, the boundary genes also varies between *Ipomoea* species and *Cuscuta* species, compared (Fig.3). The overall location of IR/SC boundaries and genes were found to be varying in Convolvulaceae and *N. tobacco* studied.

In closely related species, IR boundary shift reported being relatively minor resulting in the gain or loss of a small number of genes (Wicke et al. 2014; Downie and Jansen 2015; Wu and Chaw 2015). However, in *C cretica*, exceptional loss of genes from IR to SC regions was observed compared to other members of Convolvulacease. Many plants have lost a major portion of IR or even all, as reported for conifers, many legumes and *Erodium* species (Palmer et al. 1987; Raubeson and Jansen 1992; Guisinger et al. 2011; Guo et al. 2014).

IR boundaries expansion or contraction in SC region up to several hundred bp or by several kb which relocated multiple genes into or out IR has a great impact on plastome DNA evolution (Perry and Wolfe 2002). Although the presence of IRs in plastome is typical, few exception like some algae (Turmel et al. 2005), land plants plastomes lack IR, (Milligan et al. 1989; Cai et al. 2008) indicating that it is not essential feature for plastome function or maintenance (Guisinger et al. 2011). The observation suggests that most genomes that have lost partial or full IR exhibit extensive gene order rearrangements that the presence of the IR plays an important role in stabilizing the plastome (Palmer and Thompson 1982; Hirao et al. 2008). Similarly, lack of IR and rearrangement of plastome in *Trifolium* is reported (Cai et al. 2008).

Genes with introns

The genes with introns showed significant variations among Convolvulacae. Maximum 12 genes were found in *Ipomoea nil*, followed by 10 in *I.purpurea* and *I.batatas*, 9 in *C.cretica* and *Cuscuta reflexa*, 6 in *C.exaltata* 2 in *C.obtusiflora* and 1 in *C.gronovii* is observed (Table-4). Plastome tRNA gene sequences are generally highly conserved and loss is unusual among angiosperms with some exceptions (Morden and Wolfe 1991; Chumley et al. 2006). In *C. cretica*, loss of an intron in *trnF-AAA* and *trnG-TCC* were observed as compared to *Ipomoea nil*. Further, *C.cretica, ndhJ* showed an intron 27bp, was not found in any other members of Convolvulacease. On the other hand, an intron was lost from ycf1in *C.cretica* and *Cuscuta* species but in *Ipomoea* species, three introns were observed. In general, introns and gene loss were more common in *Cuscuta* species where in *C. gronovii* showed an intron in *clpP* gene only. Thus marked distinction in loss of introns in the genes in Convolvulacease is observed.

Overall gene arrangement on plastomes of Convolvulacease remained nearly uniform. All taxa of *Ipomoea* and *Cuscuta* lack an intact *infA* gene (McNeal et al. 2007). We have also observed 234 bp partial *infA* gene sequences in *C.cretica* support the view that this gene loss probably occurred prior to the divergence of Solanaceae from Convolvulaceae (Schmitz-Linneweber et al. 2001). The loss of *infA* from plastome has been reported in angiosperm evolution (Millen et al. 2001). In addition, *ycf15* gene is lost across Convolvulaceae (McNeal et al. 2007) but present

in *N. tobacco*. A third gene *rpl23* is a pseudo gene in *Cuscuta* and function in *Ipomoea* is not clear. The presence of *rpl23* in *C. cretica* needs tests of expression for confirmation.

In sum, an exceptional shift in IRs to SC regions is experienced in *C. cretica*. As a result, many genes shift in either SC region showed remarkable variation in nucleotides patterns. Further, the shift was not only from the IR boundaries but in between the IR regions led to segment IRs. Even the total of pieces of IRs was also 2 to 4 times less than that of any other member of Convolvulaceae compared. *C. cretica* habitat in the environmentally challenged area, the shift in IRs may be the strategic move for adaptation in this harsh environment. However, plastome analysis of some additional plants from this area may help to confirm the conclusion.

Materials and Methods

Chloroplast DNA isolation and sequencing

Chloroplast DNA was extracted from leaves of *Cressa cretica* according to Shi et al. 2012 protocol. Chloroplastic DNA was confirmed on 1% agarose gel electrophoresis and concentration was checked. About 10µg of total DNA was used for genome sequencing. Whole genome shotgun sequencing of chloroplast genome was performed using a high throughput ion torrent genome machine with ion torrent server (torrent suite v3.2).

Genome assembly and annotation

Number and quality of raw reads obtained were evaluated, checked for adapter contamination and average quality score with FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were quality trimmed using CLC Genomics Workbench v9.5.64 (CLC bio, QIAGEN) with quality score 0.05. Reference guided assembly was performed using CLC (mapping parameters: Mismatch cost=2, Insertion cost=3, Deletion cost=3, length fraction= 0.5, similarity=0.8) with the published Convolvulaceae genome of *Ipomoea nil* (NC_031159), as the reference genome. Contigs with >50× sequence depth were used for reference guided assembly. The vote majority conflict resolution mode was used in order to ensure inclusion of only chloroplast specific reads thus avoiding contribution of nuclear and mitochondrial reads to the consensus sequence. Trimmed reads were de novo assembled using CLC. Consensus sequence derived from reference assembly was compared and

corrected with de novo assembly. Plastome annotation was performed in DOGMA (Wyman et al. 2004) and CpGAVAS (Liu et al. 2012). All Gene sequences were confirmed by comparing them with available Convolvulaceae genomes and manually corrected. Further tRNA genes were confirmed using tRNAscan-SE 2.0 (Lowe and Eddy 1997).

Data collection

Complete Convolvulaceae plastome sequences of *Ipomoea batatas* (NC_026703), *Ipomoea nil* (NC_031159), *Ipomoea purpurea* (NC_009808), *Cuscuta reflexa* (NC_009766), *Cuscuta exaltata* (NC_009963), *Cuscuta gronovii* (NC_009765), *Cuscuta obtusiflora* (NC_009949) and *Nicotiana tabacum*- standard (NC_001879) were retrieved from NCBI for comparison and analysis.

Genome analysis

Gene comparison and graphical views were generated using Mauve plugin in Geneious. Further mauve (Darling et al. 2004) was used for whole genome comparative studies. ClusatlX2 (Larkin et al. 2007), Mega7 (Kumar et al. 2016) and Dnasp v5 (Librado and Rozas 2009) were used for multiple sequence alignment, computation of pairwise distance and comparative sequence analysis. Phylogenetic tree was constructed by maximum likelihood method using 100 bootstrap replicates in MEGA7. Ka/Ks ration was calculated by DnaSPV5 and PamL4.9 (Xu and Yang 2013). SNPs and nucleotide diversity was analyzed by Mauve and DnaSPV5.

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Data Accessibility:

The accession number of complete chloroplast genome of *Cressa cretica* deposited in Genbank accession: NC_035516

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Table 1 Comparative analysis of plastome data in *Cressa cretica* and other members of Convolvulaceae

Species	Size (kb)	IRa region	IRb region	LSC	SSC
Cressa cretica (Cc)	141.419	7.042	7.042	94.808	32.527
Cuscuta exaltata (Ce)	125.373	16.701	16.701	82.721	9.250
Cuscuta gronovii (Cg)	86.744	14.354	14.354	50.973	7.063
Cuscuta obtusiflora (*co*)	85.286	14.131	14.131	50.207	6.817
Cuscuta reflexa (*Cr*)	121.521	16.741	16.741	79.468	8.571
Ipomoea batatas (Ib)	161.303	20.692	20.692	87.823	32.089
Ipomoea nil (In)	161.897	30.847	30.847	88.117	12.086
Ipomoea purpurea (Ip)	162.046	30.882	30.882	88.172	12.110

Table 2 Comparative studies on genomic data of *C. Cretica* and other members of Convolvulaceae

Feature	*Cressa cretica*	*Ipomoea nil*	*Ipomoea purpurea*	*Ipomoea batatas*	*Cuscuta reflexa*	*Cuscuta obtusiflora*	*Cuscuta gronovii*	*Cuscuta exaltata*
Entire chloroplast genome size (bp)	141419	161897	162046	161303	121521	85286	86744	125373
No. of genes	121	132	131	141	113	98	98	117
No. of Proteins	82	87	85	93	69	61	62	67
No. of tRNA	27	38	38	40	35	29	28	35
No. of rRNA	8	8	8	8	8	8	8	8
No. of genes with introns	10	12	10	10	9	2	1	6
GC content (%)	38.6	37.47	37.48	37.59	38.22	37.84	37.72	38.12
GC content for gene sequences (%)	38.9	38	38.1	38.1	38.7	37.4	37.4	38.4
GC content for coding sequences (%)	39.1	38.4	38.5	38.5	39.1	37.5	37.5	38.8
GC content for rRNA genes (%)	54.5	54.9	54.9	54.7	55.2	54.7	54.7	55.3
GC content for tRNA genes (%)	52.7	49.6	51.7	51.7	52.8	50.2	51.2	52.7
NCBI accession numbers	NC_035516	NC_031159	NC_009808	NC_026703	NC_009766	NC_009949	NC_009765	NC_009963

Table 3 Comparative studies on nucleotide diversity from extended LSC and extended SSC regions due to IR loss in *C. cretica*

Parameter	IR /LSC shift	IR/SSC shift
Mean pairwise distance	0.356	0.0033
%Pair wise identity	0.860	0.98
Polymorphic sites	225	16
Nucleotide diversity pi	0.02156	0.03424
Indel sites	812	44
Haplotype/Hd	2/1	2/1
Gene shift (with or without complete identical sequences in two copies)	*trnV-GAC, ycf15,rps12,rps7,ndhB, trnL-CAA,ycf2* single copy- *trnl-CAU (*from IRa only)	*rrn4.5, rrn5,trnR-ACG,trnN-GUU,ycf1,rps15,ndhH*
Genes present in SSC in single copy		*ndhA,orf188,ndhI,ndhG,ndhE,ndhD, ccsA,psaC,ndhF,rpl32,trnL-UAG*

Table 4 Variations in number of the genes with introns in Convolvulaceae

Gene	***Cressa cretica***	***Ipomoea nil***	***Ipomoea purpurea***	***Ipomoea batatas***	***Cuscuta reflexa***	***Cuscuta obtusiflora***	***Cuscuta gronovii***	***Cuscuta exaltata***
matK	-	-	-	-	1	-	-	-
atpF	1	1	1	1	1		-	1
rpoC1	1	1	1	1	1		-	1
ycf3	2	2	2	2	2		-	2
ndhJ	1	-	-	-	-	-	-	-
accD	1	2	2	2	1	2	-	-
clpP	2	2	2	2	2		2	-
ndhB	1	1	1	1	-		-	-
ndhA	1	1	1	1	-		-	-
ndhB	1	1	1	1	-		-	-
trnF-AAA	-	2	-	-	**trnA-TGC*-1	*trnK-TTT*-1	-	**trnA-TGC*-1
trnG-Tcc	-	2	-	-	**trnA-TGC*-1		-	**trnA-TGC*-1
Ycf1	1	3	3	3	**ycf2*-1		-	-
NCBI accession numbers	MF 067398	NC_031159	NC_009808	NC_026703	NC_009766	NC_009949	NC_009765	NC_009963

*indicate different genes with intron in that plant.

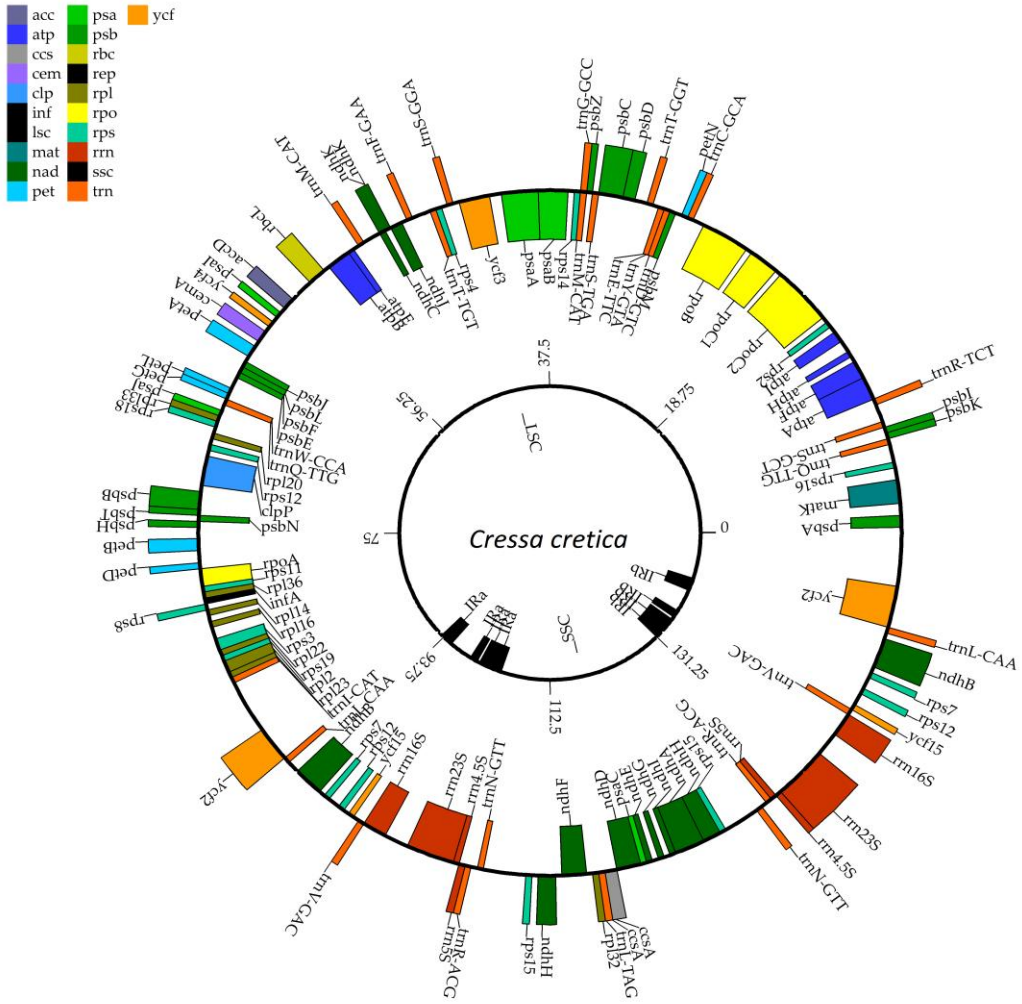
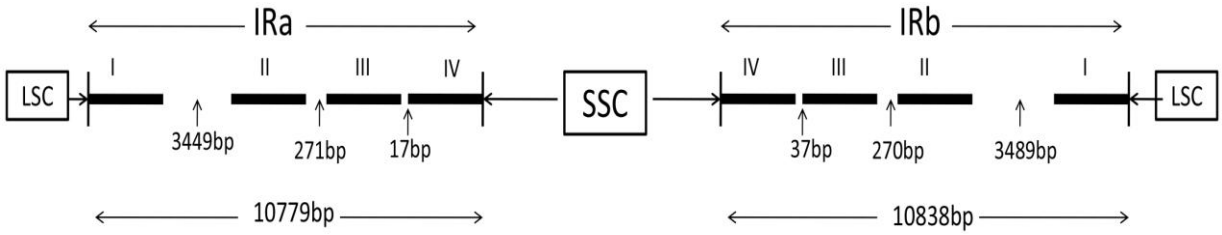
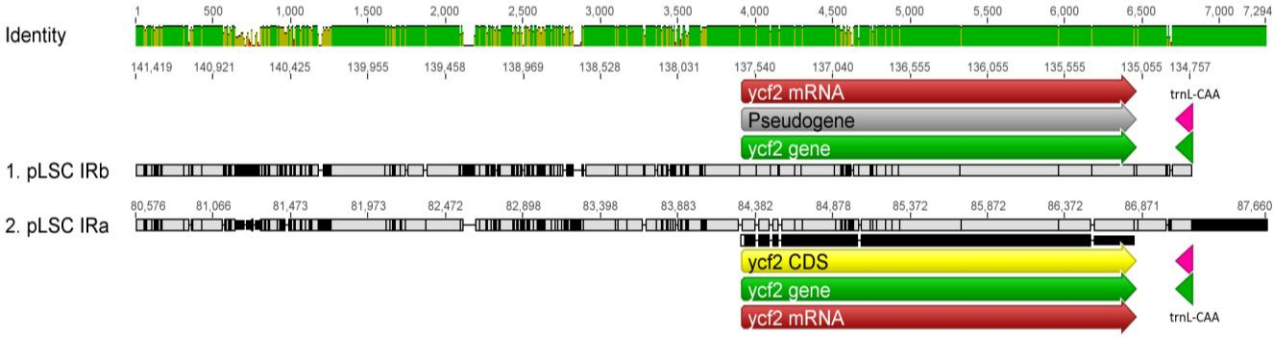


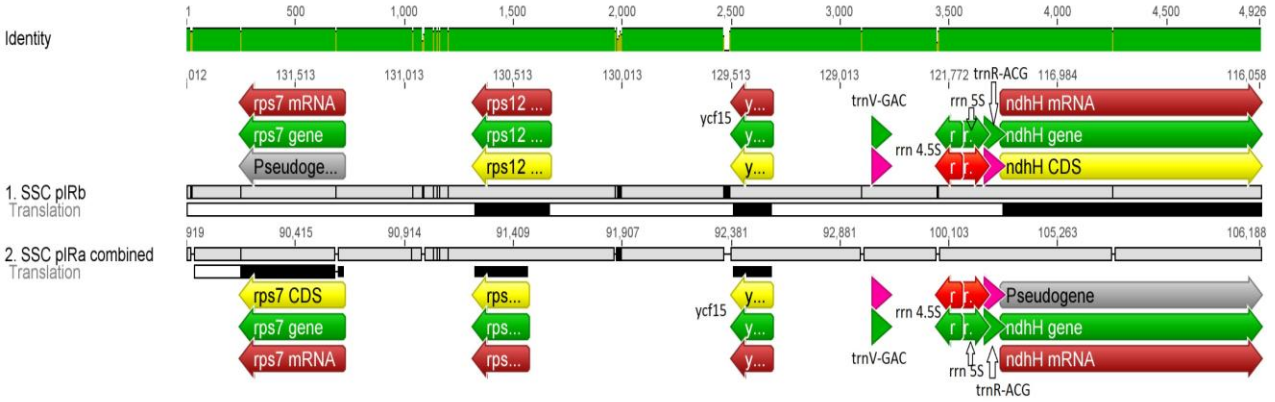
Fig. 1 Genome map of *Cressa cretica* chloroplast prepared using CPGAVAS server (Liu et al. 2012). Genes shown in the clockwise direction on outside of the structure is transcribed in anti clockwise direction while genes shown on the inside of the circle are transcribed in the clockwise direction. Legend indicates the functional group to which each gene belongs



2(a)



2(b)

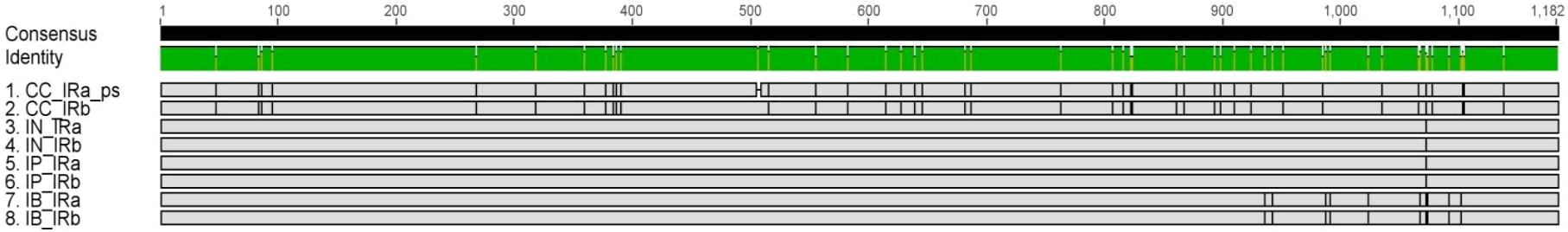


2(c)

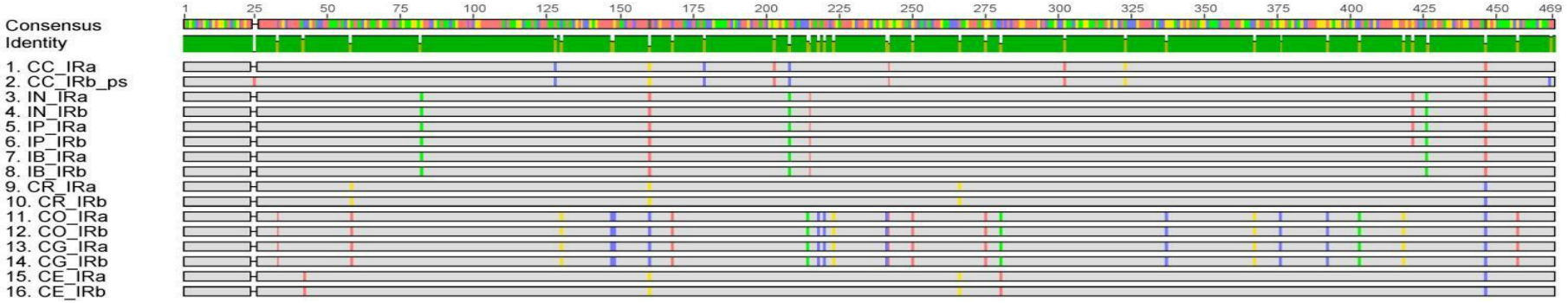
Fig. 2 (a). LSC/IR and SSC/IR boundaries on *Cressa cretica* plastome. IRaI-IV and IRbI-IV shows segmented inter repeat regions, with bp gap in between.

(b). Gene shift in LSC/IR boundaries. Broken lines indicate indels on the shifted sequences whereas solid lines indicate complete gene frame.

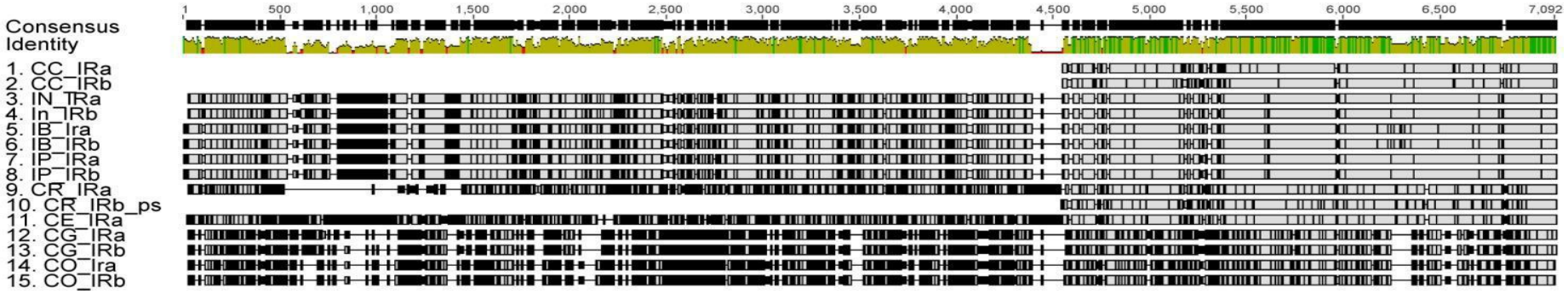
(c). Gene shift in SSC/IR boundaries; Otherwise as per fig.2b.



ndhH



rps7. The color bars shows polymorphic sites. Red:A, Green:T, Yello:G, Blue:C



ycf2

Fig. 3 Comparative alignment of shifted genes of *C. cretica* with other members of Convolvulaceae. Bars in the sequence alignment represent variations in nucleotides between and within the gene sequences. Other details are as per Table-1.

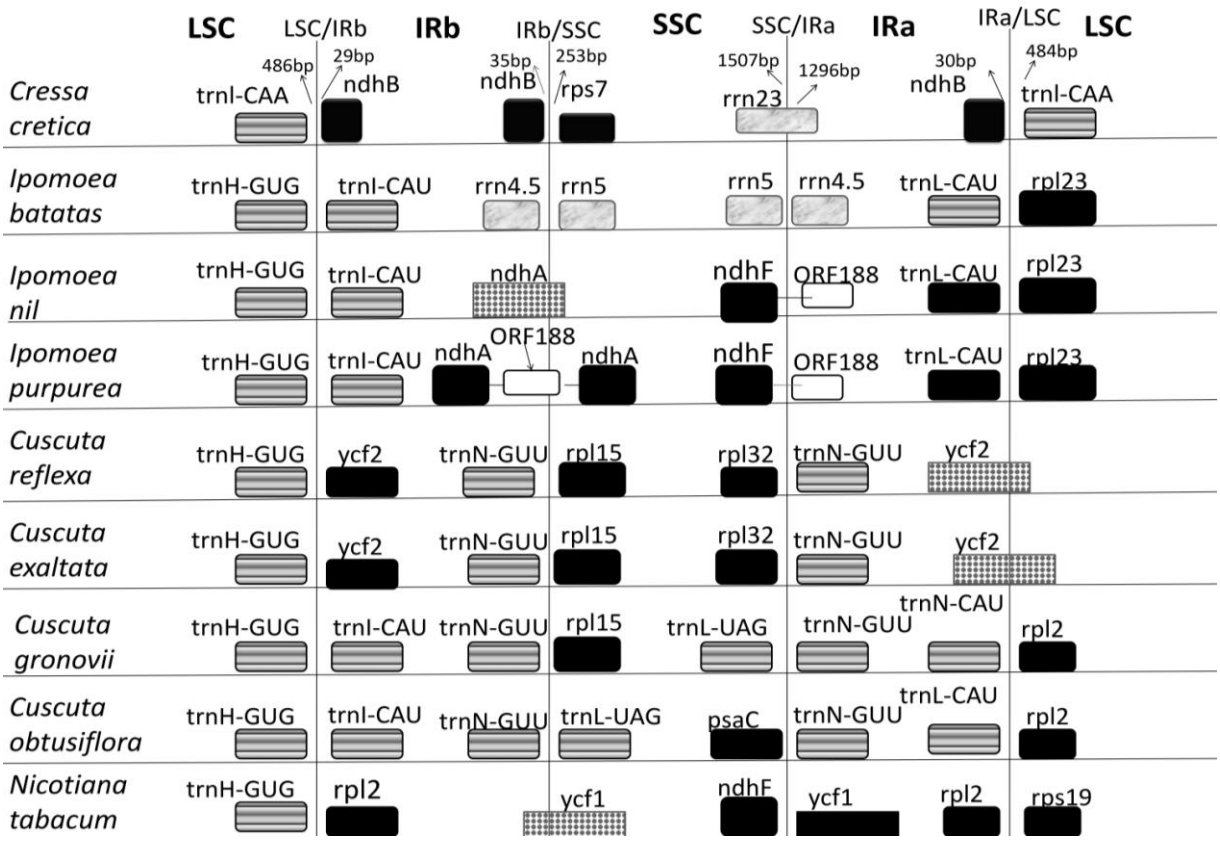


Fig. 4 Comparative structures of the LSC /IR and SSC/IR boundaries in Convolvulaceae and *N. tabacum* as out group