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Original article

The first report describes features of the chloroplast genome of *Withania frutescens*

Ahmed M Ramadan [a,b,c,*], Taimyiah Mohammed [a], Khalid M Al-Ghamdi [a], Abdullah J Alghamdi [a], Ahmed Atef [a,b]

[a] Biological Science Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia
[b] Princess Najla bint Saud Al-Saud Center for Excellence Research in Biotechnology, King Abdulaziz University, Jeddah, Saudi Arabia
[c] Plant Molecular Biology Department, Agricultural Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Giza, Egypt

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ABSTRACT

Genomic studies not only help researcher not only to identify genomic features in organisms, but also facilitate understanding of evolutionary relationships. Species in the *Withania* genus have medicinal benefits, and one of them is *Withania frutescens,* which is used to treat various diseases. This report investigates the nucleotides and genic features of chloroplast genome of *Withania frutescens* and trying to clarify the evolutionary relationship with *Withania sp* and family Solanaceae. We found that the total size of *Withania frutescens chloroplast* genome was 153.771 kb (the smallest chloroplast genome in genus Withania). A large single-copy region (91.285 kb), a small single-copy region (18.373 kb) form the genomic region, and are distinct from each other by a large inverted repeat (22.056 kb). 137 chloroplast genes are found including 4 rRNAs, 38 tRNAs and 83 protein-coding genes. The *Withania frutescens* chloroplast genome as well as four closest relatives was compared for features such as structure, nucleotide composition, simple sequence repeats (SSRs) and codon bias. Compared to other *Withania* species, *Withania frutescens* has unique characteristics. It has the smallest chloroplast genome of any *Withania* species, isoleucine is the major amino acid, and tryptophan is the minor, In addition, there are no *ycf3* and *ycf4* genes, fourth, there are only fifteen replicative genes, while in most other species there are more. Using fast minimum evolution and neighbor joining, we have reconstructed the trees to confirm the relationship with other Solanacaea species. The *Withania frutescens* chloroplast genome is submitted under accession no. ON153173.

1. Introduction

Medical plants have a major role in the production of structurally distinct phytomolecules with a range of therapeuticand economic relevance (Srivastava and Sangwan, 2020). Secondary metabolites and essential oils found in medicinal plants have significant therapeutic and health benefits. Along with their effectiveness, affordability, and accessibility, medicinal plants are said to provide other substantial benefits for treating a variety of conditions. These benefits also led to their widespread use in the treatment of microbial infections by conventional medical professionals (Upendra and Ahmed, 2021). Medicinal plants in India have been used in herbal treatments for the efficient management of a wide range of illnesses and syndromes (Ningthoujam et al., 2013). The Withania genus belongs to the Solanaceae family of flowering plants. In addition to being part of the Magnoliophyta division, it contains 23 different species found in North Africa, western and south Asia, southern Europe, and the Mediterranean region (Mirjalili et al., 2009). It is believed that Withania was named in honor of a British geologist, Henry Witham who began writing about fossil botany in 1830 (Symon, 1981). The roots of *Withania* are aphrodisiac, diuretic, aphrodisiac, astringent, bitter, acrid, somniferous, thermogenic, and stimulant. The leaves have antibacterial, anticancer, anti-inflammatory, and anti-hepatotoxic activities. The seed has hypnotic, diuretic, and milk-coagulating qualities (Bharti et al., 2016). Traditional medicine has recognized Withania as an effective treatment for stress, tuberculosis, inflam-

* Corresponding author at: Department of Biological Sciences, Faculty of Science, King Abdulaziz University (KAU), P.O. Box 80141, Jeddah 21589, Saudi Arabia.
E-mail addresses: aamara@kau.edu.sa (A.M Ramadan), kalghamdy@kau.edu.sa (T. Mohammed), tsalehmohammed@stu.kau.edu.sa (K.M Al-Ghamdi), agalghamdi1@kau.edu.sa (A.J Alghamdi), abayoumi@kau.edu.sa (A. Atef).
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mation, etc. (Archana and Namasivayam, 1998; Jayaprakasam and Nair, 2003; Bhattacharya and Muruganandam, 2003) as well as model organisms that exhibit immunomodulatory properties (Sharma et al., 2010). There are 61 species in the Withania genus that have been identified worldwide, however, only five were accepted as names: *Withania somnifera* (L.) Dunal, *Withania japonica* (Franch and Sav), *Withania coagulans* (Stocks) Dunal, *Withania begonifolia* (Roxb.) Hunz, and *Withania frutescens* (L.) Pauquy. (Srivastava and Sangwan, 2020). *Withania frutescens* (*W. frutescens*) is a perennial woody medicinal plant. Bioactive chemical classes found in this plant include polyphenols, mucilage, saponins, tannins, and flavonoids. Many indigenous people used *W. fructescens* to treat intoxication, while other reports also documented its medicinal properties (antioxidant, antimicrobial, and antifungal) (Jamal 1998; El Moussaoui et al., 2019). As far as we know, nobody has reported the toxicity of *W. frutescens* (El Moussaoui et al., 2020). Chloroplasts (cp) are found in most species of plants between 72,000 and 217000 bp in size and are one of the defining issues in evolutionary studies (Moore et al., 2007). The chloroplast genome is typically a four-part structure, having a large single copy (LSC), a small single copy (SSC), and two inverted repeats (IR) (Palmer 1991). In closely related and distantly related species, both constriction and extension of the IR can affect the size and number of genes (Ahmed et al., 2012; Abdullah et al., 2019a). Mutations occur in chloroplast genomes in a variety of ways, including insertions, substitutions, deletions, genome rearrangements, inversions, and translocations (Ahmed et al., 2012; Sloan et al., 2014; Daniell et al., 2016). Phylogenetic and taxonomic discrepancies have been resolved by exploiting polymorphism in chloroplast genomes (Ahmed et al., 2012; Sloan et al., 2014; Daniell et al., 2016; Guo et al., 2017). Furthermore, DNA barcoding can also be used for the identification of plant species (Nguyen et al., 2017; Lee et al., 2017), for chloroplast transformation to produce vaccines and metabolites, plant evolution (Wambugu et al., 2015; Waheed et al., 2015), and for the selection of cultivars to produce by selecting the most suitable taxa for breeding (Lössl and Waheed, 2011).

Numerous significant economic species belong to the Solanaceae family. The development of novel cultivars with qualities that meet market demands can be facilitated by understanding the relationship based on the chloroplast genome for all species belonging to this family (Madhav et al., 2015).

The present study continues previous efforts (Mehmood et al., 2020a,b) to determine the details of chloroplast genomes of *Withania* species, which are still largely undefined, such as expansion and contraction. The chloroplast genome sequence of *Withania frutescens* is reported here. For an evolutionary story of this genus, we compare those in four other species of *Withania*.

2. Materials and methods

2.1. Mapping of Withania frutescens cp genome

Reads quality of publicly available genomic data of *Withania frutescens* paired-end (acc.no. SRR9845561) was determined using FastQC (Andrews 2010). A large contig was formed from raw data of long reads using Velvet 1.2.10 (Zerbino and Birney 2008). Genoious R 8.1 (Kearse et al., 2012) was used to generate a complete genome from the contigs generated by velvet. IR, LSC, and SSC boundaries were assigned manually.

2.2. Withania frutescens cp genome

Withania somnifera NC_047245 was used as a reference genome for annotation. Additionally, GeSeq (Tillich et al., 2017) and CPGAVAS2 (Shi et al., 2019) were used to annotate the genome sequence. Our next step was to correct codons and coordinate intron positions, and then to compare and curate all annotations. Aragorn version 1.2.38 (Laslett and Canback 2004) and tRNAscan-SE version 2.0 were used to verify the tRNA genes.

BMW (Li and Durbin 2009) and Tablet (Milne et al., 2009) were utilized to map and visualize all reads to assembled *Withania frutescens* chloroplast genome, otherwise, circular map was created using OGDRAW (Lohse et al., 2007). *Withania frutescens*'s chloroplast genome was submitted to NCBI under acc.no. ON153173.

2.3. Comparative evaluation of the Withania frutescens chloroplast genome

Withania frutescens cp genome was compared to those of *Withania* species, including *Withania somnifera*, *Withania coagulan*, *Withania riebeckii*, and *Withania adpressa*. Geneious R8.1 was used to estimate Codon bias and amino acid (Kearse et al., 2012). The divergence regions were analyzed by mVISTA (Frazer et al., 2004) in Shuffle-LAGAN mode. Through IRscope (Amiryousefi et al., 2018), On the IR boundary between different sections of the genome (LSC/IRb/SSC/IRa) could be seen expanding and contracting. A microsatellite was measured using MicroSAtellite (MISA) (Beier et al., 2017), in mononucleotides, the threshold is 7 nucleotides, in di's, 4 nucleotides, in tri's, tetra's, penta's, and hexanucleotides, 3 nucleotides each. The phylogenetic analysis was performed by NCBI using family Solanaceae.

2.4. Phylogenetic tree

Phylogenetic tree is achieved by Blast Tree View (https://blast.ncbi.nlm.nih.gov/blast/treeview) using two methods, Fast minimum evolution and Neighbor joining with Max Seq Difference 0.75.

3. Results

3.1. Genome features of chloroplasts of Withania frutescens

Approximately 6 GB of data were generated with 5.5 million reads from the HiSeq2500 and coverage depth of 1000x in the chloroplast genome assembled from de novo. Chloroplast genome of *Withania frutescens* measures 153,771 bp and has four regions: LSC (91,285 bp), SSC (18,373 bp), and IRa and IRb (inverted repeats) each measuring approximately 22,057 bp (Fig. 1, Fig. 2). The 137 genes found in Withania frutescens cp encode 88 proteins, 4 rRNAs and 38 tRNAs (Table 1, Table S1).(Table 1, Table S1).

3.2. Gene regions and features

Introns were found in 15 of these 137 genes. Three ribosomal genes, two RNA polymerase genes, and seven respiratory coding genes have one intron, while *PafI*, *rps12*, and *clpP* each have two introns (Fig. 3, Table S2). 1138 base pairs make up the longest intron of the *ndhA*. 3′ end of rps12 is in the IR and 5′ end in the LSC. The LSC possesses 65 coding genes, SSC region 11 and the IR region 16 coding genes (4 rRNAs, 6 tRNAs and 6 protein-coding genes). GC content in the cp genome was 37 %, including 43.7 % in the IRs regions, 31.7 % in the SSCs, and 35.6 % in the LSCs. Coding regions (CDS) had GC content of 37.8 %, rRNA had 54 %, and tRNA had 56 % (Table 1).

3.3. Codon bias, nucleotide and amino acid composition

Several *Withania* species shared the same nucleotide composition and structure, including *Withania frutescens*, which was

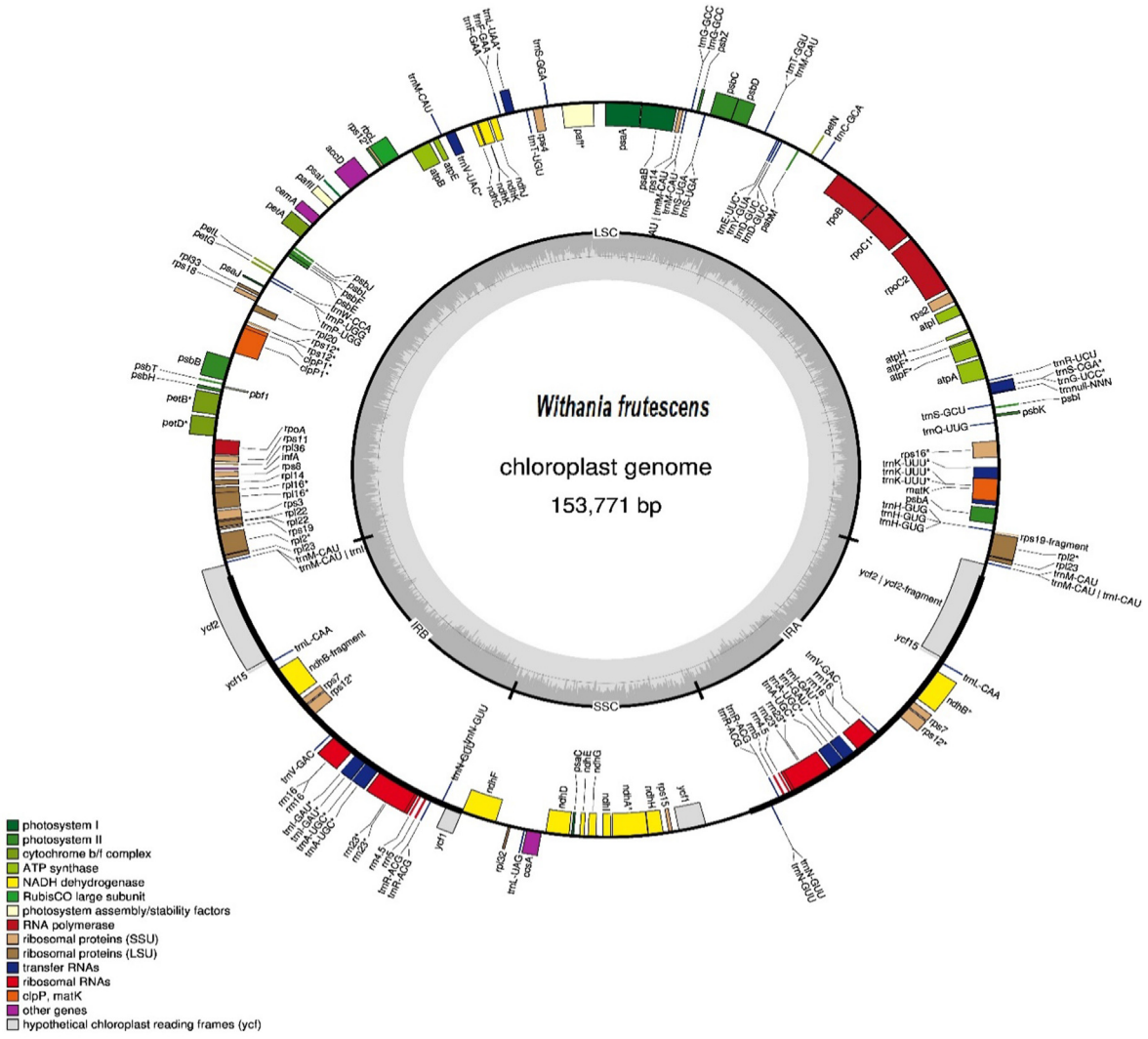

Fig. 1. *Withania frutescens* plastid genome diagram. An inner circle was drawn around the genes transcribed clockwise, and an outer circle around the genes transcribed counterclockwise. Each group of genes is the same color. Drake gray is GC content, light gray is AT content. Large single copy (LSC) and small single copy (SSC). IRa and IRb are two inverted repeat locations.

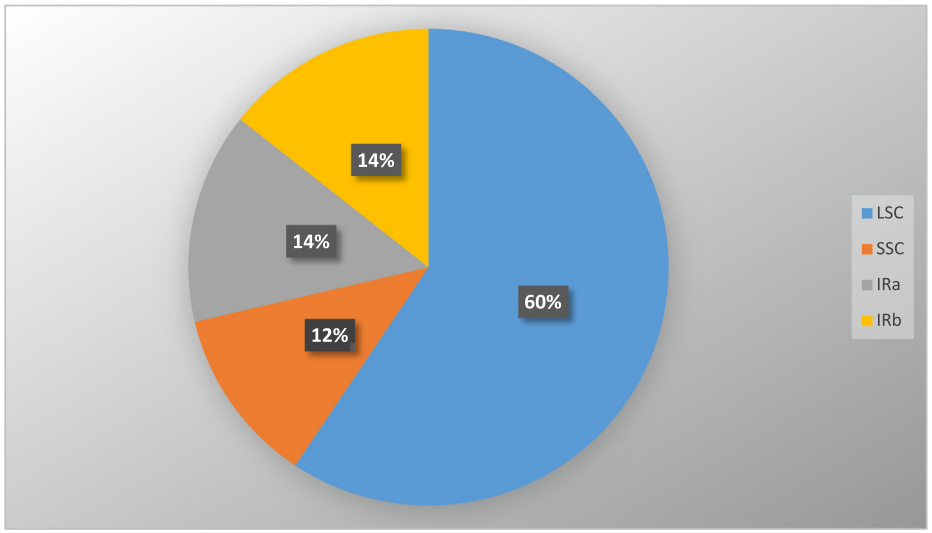

Fig. 2. The percentages of *Withania frutescens* chloroplast main region. LSC (blue), SSC (orange), IRa (gray), IRb (yellow).

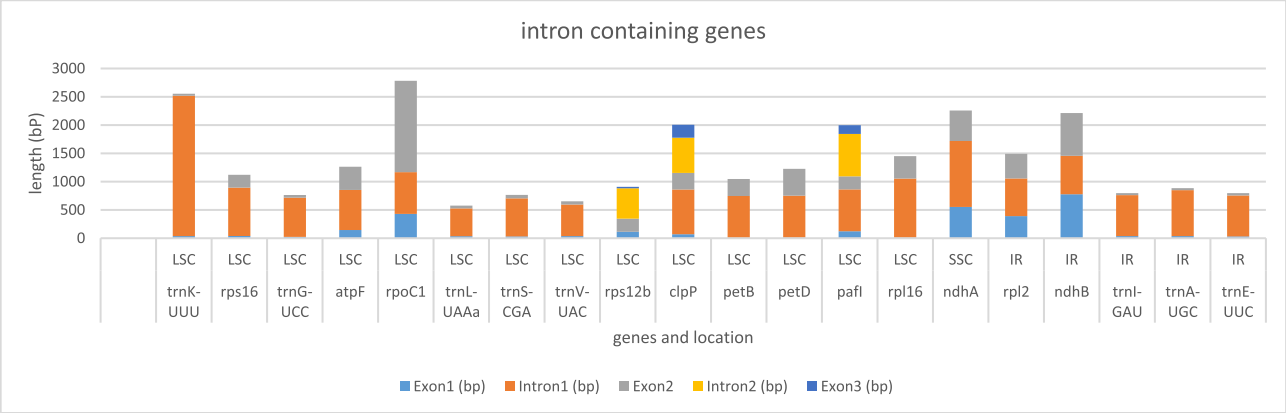

Fig. 3. The intron containing genes in *Withania frutescens* chloroplast genome including exons and introns length. Exon1 length (blue), intron1 length (orange), exon2 length (gray), intron 2 length(yellow), exon3 length(blue).

Table 1
Chloroplast genes of *Withania frutescens*.

Gene group	Gene name
ATP synthase	*atpA, atpB, atpE, atpF, atpH, atpI*
Conserved open reading frames	*Ycf1, Ycf1, Ycf 2, Ycf 2,Ycf 15, Ycf 15*
Cytochrome *b*/f complex	*petA, petB, petD, petG, petL, petN*
Envelope membrane protein	*cemA*
Large subunit of rubisco	*rbcL*
Maturase	*matK*
NADH dehydrogenase	*ndhA, ndhB, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK*
Photosystem I	*psaA, psaB, psaC, psaI, psaJ*
Photosystem II	psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbT, psbZ
protease	*clpP1*
Ribosomal RNA genes	*16S rRNA, 23S rRNA, 4.5 rRNA, 5S rRNA*
RNA polymerase subunits	*rpoA, rpoB, rpoC1, rpoC2*
Small subunit of the ribosome	*rps11, rps12, rps12, rps14, rps15, rps16, rps18, rps19, rps19,rps2, rps3, rps4, rps7, rps7, rps8*
Subunit of acetyl-CoA-carboxylase	*accD*
The large subunit of the ribosome	*rpl14, rpl16, rpl2, rpl2, rpl20, rpl22, rpl23, rpl23, rpl32, rpl33, rpl36*
Transfer RNA genes	*trnA-UGC, trnA-UGC, trnC-GCA, trnD-GUC, trnE-UUC, trnE-UUC, trnE-UUC, trnF-GAA, trnG-GCC, trnH-GUG, trnI-GAU, trnK-UUU, trnL-CAA, trnL-CAA, trnL-UAA, trnL-UAG, trnM-CAU | trnfM-CAU, trnM-CAU | trnI-CAU, trnM-CAU, trnM-CAU, trnN-GUU, trnN-GUU, trnP-UGG, trnQ-UUG, trnR-ACG, trnR-ACG, trnR-UCU, trnS-CGA, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC, trnV-GAC, trnV-UAC, trnW-CCA, trnY-GUA,*

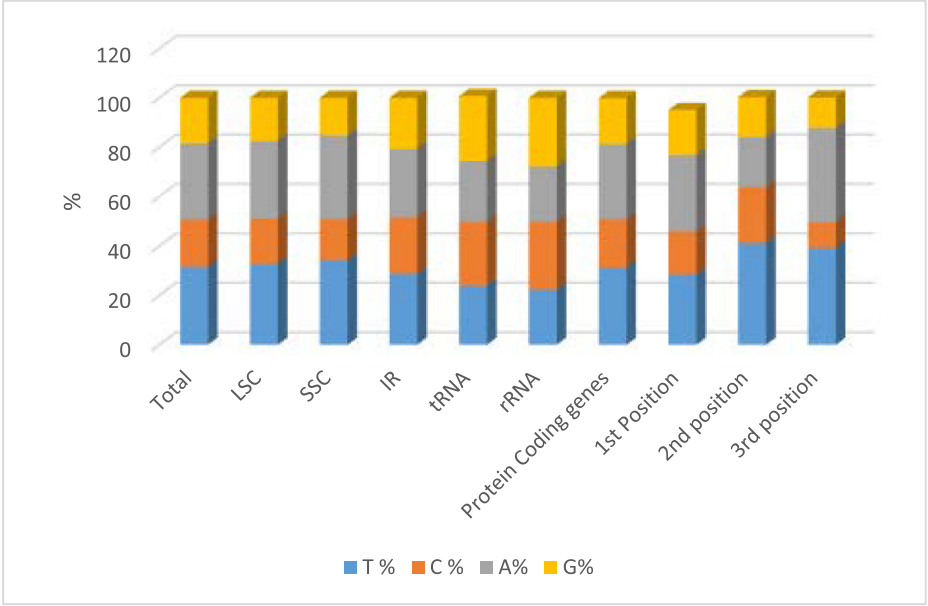

Fig. 4. Base composition in the *Withania frutescens* plastid genome. T % (blue), C % (orange), A % (gray), G % (yellow),

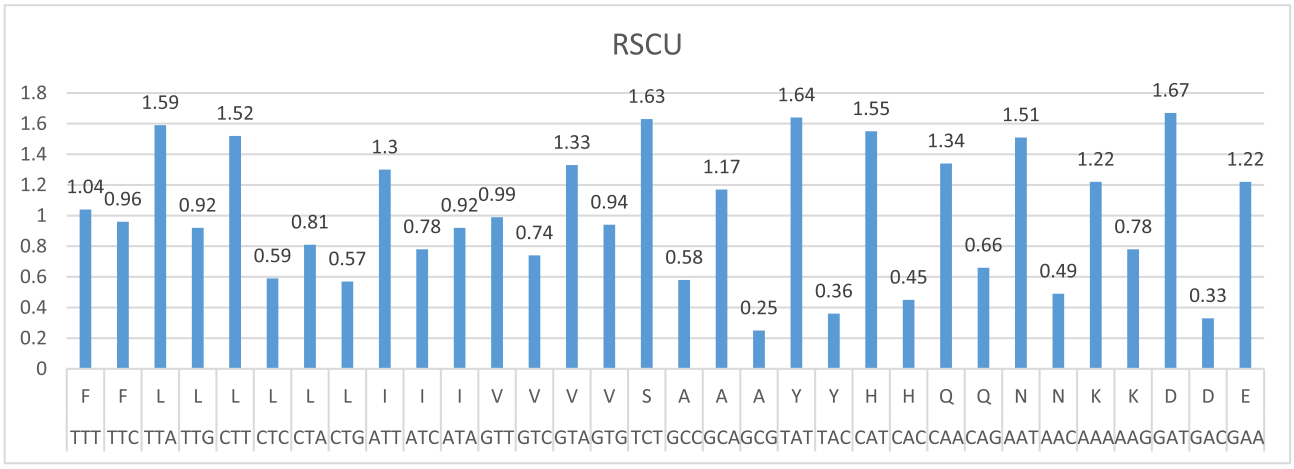

Fig. 5. Relative synonymous codon usage (RSCU) of *Withania frutescens*.

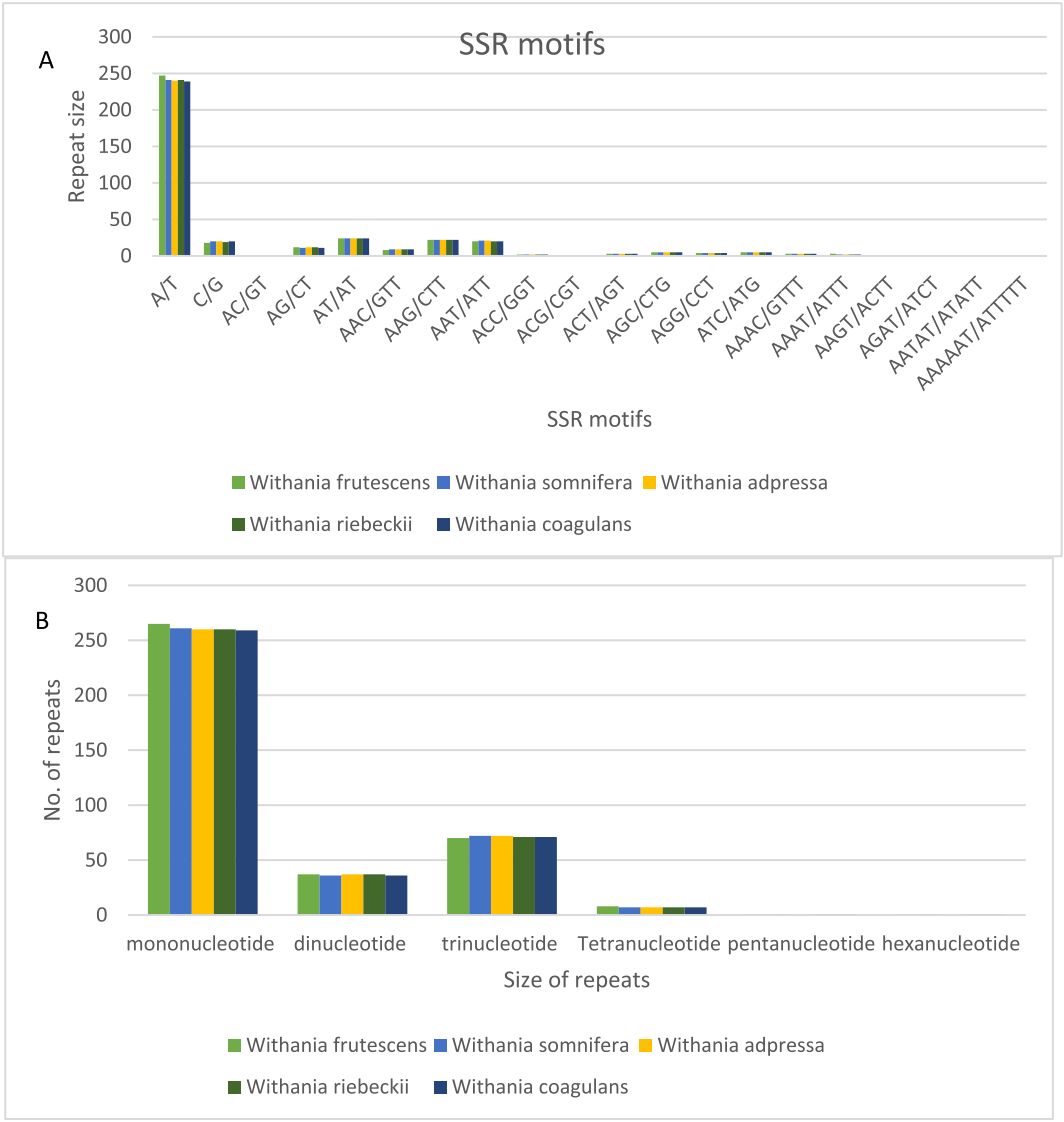

Fig. 6. Analysis of microsatellites in chloroplast genomes for *Withania frutescens*, *Withania somnifera*, *Withania coagulan*, *Withania riebeckii*, and *Withania adpressa*. *(A)* Plastid genome SSR motifs, sizes and types; *(B)* Number of various forms of SSRs.

analyzed along with four other species. The highly abundant nucleotides were Thymine and Adenine with 31.6 % and 30.7 %, respectively, while Guanine and Cytosine accounted for 18.5 % and 19.2 %, respectively. At the first (59.3 %), second (61.53 %) and third (77.38 %) positions of codons in coding sequences, the AT content was equivalent (Fig. 4. And Table S3).

Based on RSCU analysis, isoleucine was the major frequency while tryptophan was the lowest. Other *Withania* species have different chloroplast genome patterns (Fig. 5, Table. S4). Accordingly in Table S4, codons with third base A/T were favored because RSCU value > 1, while codons with third base G/C were non-favored because RSCU value < 1. These results are common in *Withania* species.

3.4. SSRs analysis

Of total 382 SSRs, Two hundred and sixty-five mono-, 37 di-, seventy-three tri-, eight tetra-, one penta-, and one hexanucleotide repetitions were observed. SSRs were distributed as follows: 238 SSRs were in LSC regions (62.3 %), 62 SSRs were in SSC regions (16.2 %) and 82 SSRs were in the IR region (22.5 %). Compared to selected species of Withania, which ranged from 375 (*Withania somnifera*) to 399 (*Withania adpressa*), there was a high level of similarity in SSRs. A high proportion of A (30.4 %, 27.8 %, 29.5 %, 29.4 % and 29.4 %) and T (34.2 %, 35.3 %, 34.4 %, 34.4 % and 34.1 %) nucleotides were detected in *Withania frutescens*, *Withania somnifera*, *Withania coagulan*, *Withania riebeckii* and *Withania adpressa*, respectively. As shown in Fig. 2, table S5 and table S6, the mononucleotides were the major (A/T repeats were highly frequency), trinucleotides (with a high number of AAG/CTT repeats) and dinucleotides (AT/TA repeats were highly frequency), however, pentanucleotides and hexanucleotides were infrequent (Fig. 6).

3.5. Extension and constriction of IR region

The barriers between LSCs, IRs and SSCs of four *Withania* species were compared comprehensively (Fig. 7). *Withania frutescens* contains the ycf2 gene at the junction between the LSC and IRb and a portion of it is found in the IRb. However, other *Withania* species the *rps19* gene located in the same region. SSC and IRb junctions contained the *ycf1* gene which had been integrated from the 5′ end of the IRb region to leave a truncated copy that was present within SSC/IRb junctions in *Withania coagulan, Withania riebeckii* and *Withania adpressa* but not found in *Withania somnifer* and found just at IRb in *Withania frutescens.* In addition, the *ndhF* gene has been found in all plants at the SSC region start, but it stretches into IRa in *Withania frutescens*.

3.6. Analyses of the Solanaceae phylogeny

In reconstructed phylogenetic trees for 20 species of the Solanaceae family including five *Withania* species, 99 % of sites were constant. However, about one percent of the nucleotide sites between species are polymorphic. All nodes of the phylogenetic tree support complete lineage sorting (Fig. 8). Fast minimum evolution and Neighbor joining results revealed that *Withania frutescens* was a sister plant to other *Withania* species.

4. Discussion

The comparison between the chloroplast genomes of *Withania sp* rich knowledge of the cp genomes' evolutionary dynamics. These studies will enable us to learn more about how genomes operate and how species like Wthania evolved. Additionally, microsatellite markers could be developed using SSRs identified in the current study. There is almost similar gene organization and gene contents in the chloroplast genomes of angiosperms across different plant lineages and from the same family of plants (Chevenet et al., 2006; Amiryousefi et al., 2018; Abdullah et al., 2019a). Cp genome of *Withania frutescens* is investigated in this study for the first time. In the first observation, we found that the size of chloroplast genomes of different *Withinia* species varied between 9 and 200 bp, but *Withania frutescens*' full-length chloroplast genome was about 600 bp smaller than *Withania somnifera*'s (Table S1). According to Mehmood et al., (Mehmood et al., 2020) and to our knowledge, we thought the chloroplast genome size of *Withania frutescens* is one of the smallest chloroplast genomes in the family Solanaceae, along with *Solanum capsicoides* and *Sola-*

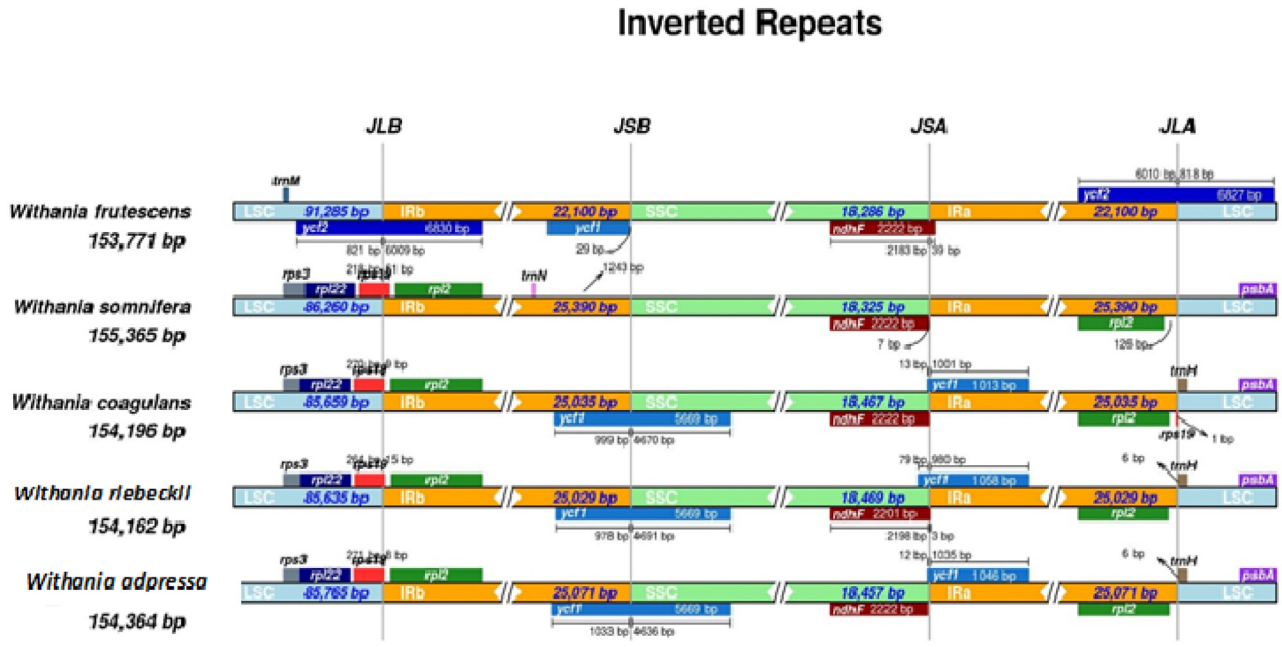

Fig. 7. Comparing the IR, SSC, and LSC regions of five plastid genomes. The top of a line shows positive strand genes, while the bottom of the line shows negative strand genes. Each gene is denoted by a box, and its length in each subregion is displayed above the box. Genes are listed with a number of base pairs to indicate whether a gene is present in one or two regions of the chloroplast genome. There are four junction sites: JLA; IRa/LSC, JSB; IRb/SSC, JLB; IRb/LSC and JSA; SSC/IRa.

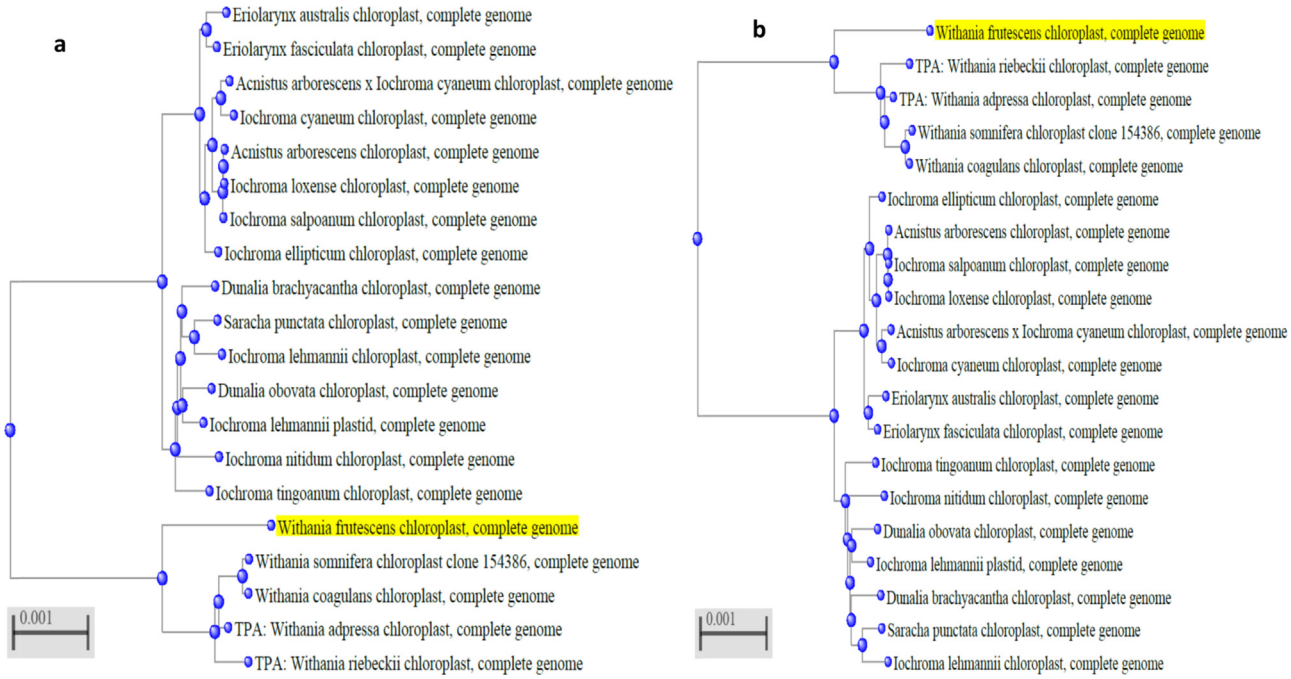

Fig. 8. Phylogeny tree is reconstructed with 20 chloroplast genomes of Solanaceae including *Withania frutescens* with two methods; a, Fast minimum evolution tree and b, Neighbor joining tree.

num amotapense. The variation of GC content between *Withania sp*. Is almost non-existent (table S1) but it varies between the regions of the same species chloroplast (table S3). This result accepts previous reports that find the GC content of chloroplast genomes varies across different regions, but it is higher in the IR regions because rRNAs contain a high amount of GC (Abdullah et al., 2019a; Chevenet et al., 2006; Kumar et al., 2018; Abdullah et al., 2019b). In addition, almost all five species of *Withania* have a similar gene content and genome structure, and the difference in gene count is due to the absence of psedogenes, such as *Ycf3* and *Ycf4*, which are present in most solanaceans (Mehmood et al., 2020) but not in *Withania frutescens. Also,* the repetition of some genes is different between species. 15 genes in *Withania frutescens,* 17 in *Withania somnifera* and 18 in other species (Table S1). Codon bias can help us learn about evolutionary processes, genome structure and selection pressures on genes (Yang et al., 2014), but the five *Withania* species showed similar codon bias patterns, demonstrating that these species' ecological niches may have been subjected to similar environmental stresses. In the genomes of *Withania*, we discovered a preference for codons that end in A or T (Table S3, S4). It is not limited to *Withania* alone, but also to most species in the family Solanaceae (Mehmood et al., 2020). These could be due to the abundance of A or T nucleotides in other angiosperm chloroplast genomes (Kumar et al., 2018; Abdullah et al., 2019a, Zhou et al., 2021).

Among Withania's chloroplast genome, SSRs and oligonucleotide repeats revealed similarities. SSRs, however, have been observed in plants from similar lineages in previous studies (Kumar et al., 2018; Abdullah et al., 2019a,b). Mononucleotide SSRs predominate over dinucleotide and trinucleotide SSRs, while A/T SSRs on mononucleotides and AT/TA SSRs on dinucleotides are abundant (Table S5, S6). These patterns of SSRs are found in Solanaceae chloroplast genomes (Amiryousefi et al., 2018) as well as in many angiosperm families (Abdullah et al., 2019a, b; Yang et al., 2014; Choi et al., 2016). Also, there were severe similarities in chloroplast SSRs between *Withania* species. However, we thought mononucleotide A/T SSRs could be used to distinguish between *Withania* species and needed more genetic studies.

In chloroplast genomes, IR shrinkage and extension are regular occurrences. These differences can occur in both closely related and distantly related species, and can result in the creation of pseudo-genes, gene duplication, and gene deletion (Kim et al., 2017; Abdullah et al., 2019a; Menezes et al., 2018). The relationship between LSC, SSC, and IR in the five species studied revealed some parallels and differences. The pseudo-gene copies of certain genes, such as ycf1, were conserved across all species except *Withania somnifera* (Fig. 7; Mehmood et al., 2020). In certain species (*Withania adpressa*, *Withania coagulans*, and *Withania riebeckii*), they are spread out in two regions (IRb, SSC), but with *Withania frutescens*, they remain in one area (Fig. 7). This phenomenon raises questions about the evolution of this species. These differences are critical to gain a better knowledge of the chloroplast genome's evolution and organization (Kim et al., 2017; Abdullah et al., 2019a).

A phylogenetic tree was constructed based on the nucleotide sequence for the Solanaceae family, and the results showed close links among Withania species. Based on a phylogenetic analysis of the chloroplast genome sequences, our results are confirmed.

In conclusion, as a first report, features of *Withania frutescens* chloroplast genome was decoded. *Withania frutescens* differs from other Withania species in a number of ways. There is no *Withania* species with a smaller chloroplast genome than this one, isoleucine is the major amino acid, and tryptophan is the least. Third, there are no ycf3 and ycf4 genes. Fourth, there are only fifteen replicative genes, while in most other species there are more. Our results may help in species identification, clarification of taxonomic ambiguities, and inference of genus *Withania* relationships. The entire cp genome sequence of *Withania frutescens* is now available, which could help researchers identify the most suitable sites for chloroplast transformation vectors and transgene integration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103600.

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