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Original Article

An insight on the complete chloroplast genome of *Gomphocarpus siniacus* and *Duvalia velutina*, Asclepiadoideae (Apocynaceae)

Uma visão sobre o genoma completo do cloroplasto de *Gomphocarpus siniacus* e *Duvalia velutina*, Asclepiadoideae (Apocynaceae)

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Abstract

We studied the complete chloroplast genome of *Gomphocarpus siniacus and Duvalia velutina* from Asclepiadoideae subfamily; due to their medicinal importance and distribution worldwide their interest became high. In this study we analyzed the complete chloroplast genomes of *G. siniacus* and *D. velutina* using Illumina sequencing technology. The sequences were compared with the other species from Apocynaceae family. The complete genome of *G. siniacus* is 162,570 bp while *D. velutina* has154, 478 bp in length. Both genomes consist of 119 genes; encode 31 tRNA genes, and eight rRNA genes. Comparative studies of the two genomes showed variations in SSR markers in which *G. siniacus* possesses 223 while *D. velutina* has 186. This could be used for barcoding in order to aid in easy identification of the species. Phylogenetic analysis on the other hand reaffirms the tribal position of *G. siniacus* in Asclepiadeae and *D. velutina* in Ceropegieae. These findings could be used in subsequent research studies of angiosperms identification, genetic engineering, herb genomics and phylogenomic studies of Apocynaceae family.

Keywords: chloroplast genomics, *Duvalia velutina*, evolution, *Gomphocarpus siniacus*, phylogenetic tree.

Resumo

Estudamos o genoma completo do cloroplasto de *Gomphocarpus siniacus* e *Duvalia velutina* da subfamília Asclepiadoideae. Em razão de sua importância medicinal e distribuição em todo o mundo, o seu interesse tornou-se elevado. Neste estudo, analisamos os genomas completos de cloroplastos de *G. siniacus* e *D. velutina* usando a tecnologia de sequenciamento Illumina. As sequências foram comparadas com as demais espécies da família Apocynaceae. O genoma completo de *G. siniacus* tem 162.570 pb, enquanto *D. velutina* tem 154.478 pb de comprimento. Ambos os genomas consistem em 119 genes e codificam 31 genes de tRNA e 8 genes de rRNA. Estudos comparativos dos dois genomas mostraram variações nos marcadores SSR em que *G. siniacus* possui 223, enquanto D. velutina possui 186. Isso poderia ser usado para código de barras para facilitar a identificação das espécies. A análise filogenética, por outro lado, reafirma a posição tribal de *G. siniacus* em Asclepiadeae e *D. velutina* em Ceropegieae. Esses achados poderão ser utilizados em pesquisas posteriores de identificação de angiospermas, engenharia genética, genômica de ervas e estudos filogenômicos da família Apocynaceae.

Palavras-chave: genômica de cloroplastos, *Duvalia velutina*, evolução, *Gomphocarpus siniacus*, árvore filogenética.

1. Introduction

Duvalia velutina (Lavranos) is a succulent, perennial plant distributed in Saudi Arabia, South Africa, Yemen and Namibia. It had distinct floral and stem characteristics which are common in almost all the species as such can only be distinguished when flowers appeared. *D. velutina* usually grow in colonies and it is locally used as food and medicine as well as ornamental purposes (Burkill, 2004). *Gomphocarpus siniacus* (Boiss) is an herbaceous plant which is found in Africa, Saudi Arabia, Egypt (Sinai Peninsula), Jordan, and Yemen. In Africa, *G. siniacus* is used for several medicinal and other uses, such as treatment of bile, tuberculosis and stomach pain. Many active compounds have been reported in these plants for example in *G. siniacus* contains some cardio-active glycosides and alkaloids (Burkill, 2004). Modern pharmacological investigations also showed their possible anti oxidative

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and antibacterial potentials. Similarly, *D. velutina* and *G. siniacus* belongs to subfamily Asclepiadoideae in the family Apocynaceae. Although previous studies on *D. velutina* and *G. siniacus* focuses on eco-physiology with few studies on molecular genetics (Lang et al., 2018; Masrahi, 2015). Also, so many attentions have been given to cultivated variety of *D. velutina* and *G. siniacus* as a result the wild varieties has been neglected; among the *Gomphocarpus* sp. only *G. siniacus* is native to Arabian Peninsula therefore it is very important for conservation and pharmaceutical discovery as well as the evolutionary studies. Majority of Asclepiadoideae species do not have a common character unique to their clades which results in wrong identifications (Abba et al., 2020).

There is need for effective molecular markers to address problem of identification in Asclepiadoideae. Some of the markers used to identify *D. velutina and G. siniacus* were ITS, *psbA, matK* and *rbcL* which has helped to some extent in the recent taxonomy of Apocynaceae (Masrahi, 2015). Due to the important economic and medicinal benefits of these species there have been adulterants stocks being sold to people in place of the original species which can affect the quality and medicinal efficacy of the target drugs and or concoctions. For this reason a correct identification of these species will greatly protect the genetic resources and lineage history. The widely used method of identifying these species was ITS and multiple genomic partial segment bar-coding (Masrahi, 2015; Lang et al., 2018). The commonly used plastid regions were *PsbA, trnH* and *matK* are not enough in the correct identification of some angiosperms (Cui et al., 2019). Chloroplast as one of the major differences between plant cells and animal cell plays a crucial role in providing energy for plants metabolism (Li et al., 2013; Neuhaus and Emes, 2000; Rodríguez-Ezpeleta et al., 2005). For many decades and now chloroplast genomes have been given many attention by plant researchers. Quadripartite structure is the main symbol of chloroplast genome and a 115 kb – 165 kb sequence length with large single-copy region (LSC), a small single-copy region (SSC) and a pair of inverted repeats regions IRa and IRb (Dong et al., 2012; Jansen et al., 2005). Chloroplast (cp) genome is very conserved in terms of its gene order and contents compared to other organelles in majority of angiosperms (Tonti-Filippini et al., 2017; Wicke et al., 2011). With that reason the evolutionary history details of most angiosperms express a unique and vital information for plant phylogeny (Corriveau and Coleman, 1988). Several research on DNA-bar-coding as well as the molecular markers give more emphasis on the chloroplast genome after the work *Nicotina tabacum* by Shinozaki et al. (1986). Many plant biologist were able to identify over 3000 plant chloroplast genomes and stored in the GenBank (Mocan et al., 2014; NCBI). Moreover, the comparative chloroplast genomes of two Asclepiadoideae species will reveal the Phylogenetic lineage between the two species and their positions in Asclepiadoideae well as the tribal positions.

The characterization of highly variable regions would contribute to developing candidate DNA barcodes for future studies. Microsatellites (SSRs) could be used as potential molecular polymorphic markers to reveal the genetic diversity and population structure of Apocynaceae. The detection of protein-coding genes under intense selection pressure could play an important role in the analyses of evolution and adaptation of plants in an ecosystem. in addition, this study would reconstruct the intergeneric relationships and locate the phylogenetic position of sub family Asclepiadoideae.

2. Materials and Methods

2.1. Plant sampling, DNA extractions and sequencing

Fresh leaves of *D. velutina* and *G. siniacus* were obtained from the Ash-shafa Mountains in At-Taif city of Makkah Region, Saudi Arabia (21° 4.7'33'N; 41°17.9' 29" E) on the 2nd of June 2019. Samples were identified at the herbarium of Department of Biological Sciences, Faculty of Science of King Abdulaziz University Jeddah KSA. The voucher specimens of the two plants species were deposited at the herbarium of King Abdulaziz University, Jeddah. The leaves were washed with 70% ethanol and then DNA was extracted using DNeasy Plant mini kit following standard protocol (Qiagen Co. Germany). Quality of the DNA was checked using Nanodrop 2000C Spectrophotometer and Electrophoresis in 1% (w/v) agarose gel. The pure DNA was used to construct the libraries and was sequenced with Illumina Hiseq 2500 (Beijing, China) following the standard protocol. The result of 6.8 Gb of *D. velutina* and 7.1 *G. siniacus* and pair reads of 500 bp were recovered.

2.2. Chloroplast genome assembly and annotation

The Raw sequence was filtered using Skewer 0.2.2 and trimmed with Trimmomatic V).36 (Bolger et al., 2014). Sequences were mapped with the reference genome from the NCBI using BLASTN with default settings. In which the *Stapelia gigantea* (MG963259) complete chloroplast genome was used as reference to assemble the *D. velutina* while *Cynanchum wilfordii* (KT220734) was used to assemble the *G. siniacu*s genome. SOAPdenovo (Xie et al., 2014) was applied to assemble the contigs while the annotation was done using PGA software (Qu et al., 2019).

Geseq was used to annotate genes (Lohse et al., 2007), while ARAGORN V 1.2.2 and tRNAscan-SE V 2.0.3 was used for the annotation of tRNAs in the sequences (Lowe and Chan, 2016). For circular genome structure we use Organellar Genome DRAW (Tillich et al., 2017). The annotated sequences of *D. velutina* and *G. siniacus* were submitted to National center for Biotechnology Information (NCBI) and were assigned with an accession Numbers MT431578 and MN689141 for *D. velutina* and *G. siniacus* respectively.

2.3. Comparative analysis and genomic features

Comparative genomics was made using mVISTA programme (Mayor et al., 2000); to compare *D. velutina and G. siniacus*, sequences with *Calotropis procera (*NC_041440) and *Gymnema sylvestre (*NC_047175) genomes both from Asclepiadoideae downloaded from GenBank database.

While the expansion and contractions of the sequences were done using IR scope (Amiryousefi et al., 2018).

2.4. Amino acid frequency, codon usage, and RNA editing sites

For the sequence analysis we use MEGA 6.0 (Kumar et al., 2008) to detect the relative synonymous codon usage (RSCU), codon usage as well as the base compositions; while RNA editing sites in the protein coding genes were analyzed with PREP suite (Mower, 2009) with 0.8 cutoff values.

2.5. Microsatellites analysis

Microsatellites in the sequences of *G. siniacus* and *D. velutina* were evaluated with MISA (IPGCPR, Gatersleben, Germany) (Thiel et al., 2003) with the settings of 10, 5, 4, 3, 3and 3to represent mono, di tri, tetra, penta and hexa values respectively; while Tandem Repeats were identified with a program called Tandem repeat Finder (NY, USA) with ten base pairs length. While setting 2, 7, 7, for match, mismatch and indels respectively. The size of the repeats were viewed with program REPUter (Beilfeild Germany) (Kurtz et al., 2001) parameters were set at 30 base pairs as least size and ninety percent limit similarity index of two repeat copies.

2.6. Substitutions and InDel analyses

In order to determined substitution rates in *D. velutina and G. siniacus*, the sequence of *Gymnema sylvestre* was used as a reference (Yaradua et al., 2019) while alignment of SSC, LSC and IR regions in the genomes was done using MAFFTv.5 (Multiple Alignment with Fast Fourier Transform) (Katoh and Standley, 2016). The numbers and types of substitutions were described in Geneious R8.1(Kearse et al., 2012). InDels events were determined after analyzing a pairwise alignment the SSC, LSC and IR in DnaSP v.5.10 (Librado and Rozas, 2009).

2.7. Phylogenetic analysis

Complete plastome genome of *D. velutina* and *G. siniacus* along with other 9 species from Asclepiadoideae subfamily. Two species were outgroup from Rauvofluideae subfamily; were downloaded from the Genbank and aligned with MAFFT program v.7 (Katoh and Standley, 2013). Aligned sequences were further analyzed with Maximum Parsimony PAUP ver. 4.0b 10 (Felsenstein, 1978) with 1000 replicate tree bisection-reconnection, branch swapping, and random taxon addition; with MulTrees on and trees saving of 100 as peak value for all replicates. Missing characters were considered as a gap while support was determined using 1000 replicates nonparametric bootstrap method. Program MrBayes 3.2.6 (Ronquist et al., 2012) was employed to perform Bayesian analysis. jModelTest 3.7 (Ebert and Peakall, 2009) was used to select the right model.

3. Result

3.1. Characterization of the cp genomes of G. siniacus and D. velutina

The complete chloroplast genomes of *G. siniacus and D. velutina* were 154,478 bp and 162,570 bp in size respectively (Table 1 and Figure 1). The two genomes consist of a pair of inverted repeats (25,633 and 26,264 bp); LSC (92,547 and 84,170 bp); SSC (18,757 and 17,780 bp) for

Table 1. Characteristics of *G. siniacus* and *D. velutina* chloroplast genomes.

	G. siniacus				
	T (U) (%)	**C (%)**	**A (%)**	**G (%)**	**Length (bp)**
LSC	30.4	19.6	31.4	16.6	92,547
SSC	32.8	16.7	34.0	16.5	18,757
IRA	27.3	21.6	30.2	20.9	25,633
IRB	27.3	21.9	28.2	22.6	25,633
1st Position	32	18.6	31.0	18.6	54202
2nd Position	31	19.0	30.7	19.3	54202
3rd Position	31	19.2	30.9	18.7	54202
	D. velutina				
LSC	30	19.8	30.5	19.7	84,170
SSC	32	17.1	32.8	18.1	17,780
IRA	29	18.6	33.8	18.1	26,264
IRB	29	19.8	31.0	20.3	26,264
1st Position	30.7081773	18.4506176	32.2601681	18.581037	53673
2nd Position	31.4757886	18.7803924	30.4342966	19.3095225	53673
3rd Position	30.3640632	18.706216	31.1968997	19.7328216	53672

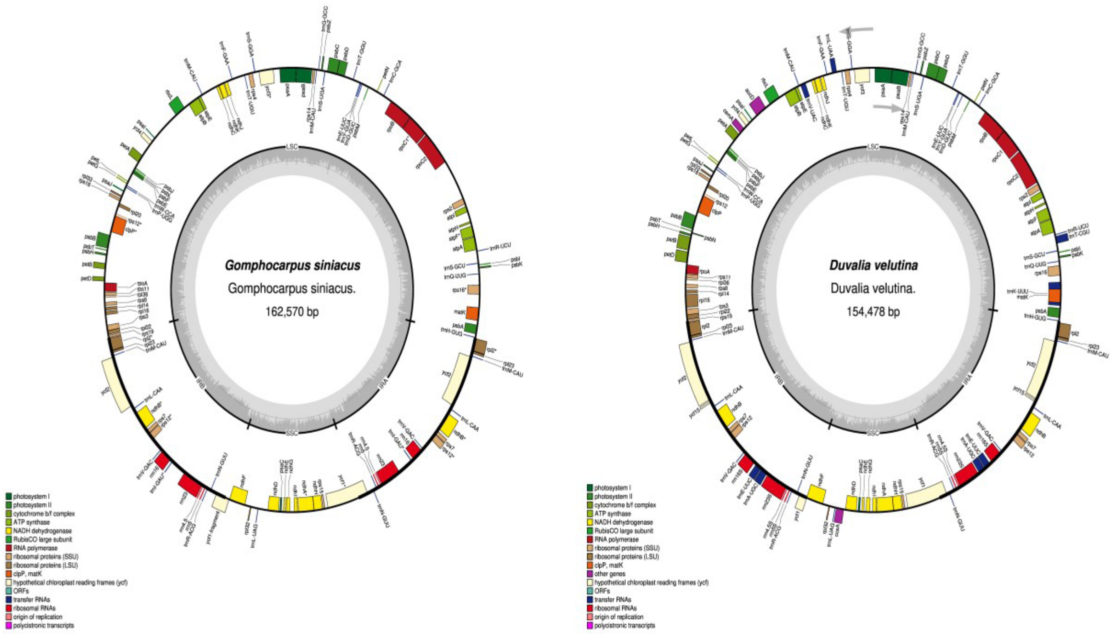

Figure 1. Chloroplast genome draw of *G. siniacus* and *D. velutina* showing gene map.

D. velutina and *G. siniacus* respectively (Table 1). Overall GC content of *Duvalia velutina* is 37.9% while *Gomphocarpus siniacus* has 38%, while the inverted repeats regions has high GC content ranges from 35.2% to 33.2%. The genome of *D. velutina* was found to be almost divided into two equal parts between the coding regions and non-coding regions.

The genomes consist of 119 genes (Table 2). The two genomes encode 31tRNA genes, 4 rRNA genes and three pseudo genes (*rps19, ycf1* and *ycf15*). Most of the genes were found to be duplicated at the IR regions of the genomes.

Predictive RNA editing sites indicate high probability in *ndhB* (8 in D. velutina and 7 in G. siniacus) and *rpoB* (7 in Both *D. velutina* and *G. siniacus*) while *matK* in *D. velutina* (3 sites) and *ndhA* in *G. siniacus* (5 sites). The conversion rate observed tend to be higher at the initial nucleotides with almost three times than the second nucleotide (Figure 2). Majority of the RNA editing sites were coding for the conversion of serine to Leucine with higher possibility of hydrophobic amino acid valine, phenylalanine, methionine isoleucine and many more. A total of 37 protein coding genes in *D. velutina* and *G. siniacus* were predicted for RNA-editing sites 19 were predicted in *D. velutina* while 21 were predicted in *G. siniacus*. Total of 8 and 6 genes do not have RNA editing sites in *D. velutina* and *G siniacus* chloroplast genomes respectively. The amino acid conversion indicate high Serine to Leucine conversion(S-L)(26), followed by Proline to Leucin (9) and proline to serine (4) and the least conversion were T-L, T-I, T-A and H-Y each with only one potential conversion Figure 2.

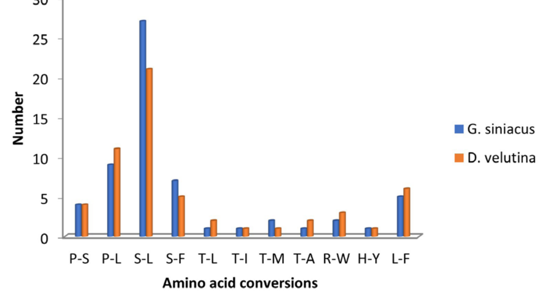

Figure 2. RNA editing amino acid conversions in *G. siniacus and D. velutina*.

3.2. Analysis of cpSSR

Analysis of cpSSR in the sequences of *G. siniacus, D. velutina, G. sylvestre* and *C. procera* chloroplast genome (Figure 3 and 4) indicates higher mononucleotide in all the sequences (31 – 99). In *G. siniacus* tetra-nucleotide are the second highest (13), di-nucleotide (12), tri-nucleotide (10), penta-nucleotide (9) and hexa-nucleotide (1); *D. velutina* second highest was tri-nucleotide (9), di-nucleotide (5), tetra-nucleotide (3), penta-nucleotide (2) and hexa-nucleotide (1); *G. sylvestre* di-nucleotide and tetra-nucleotide were second highest with (3 each), tri-nucleotide (2), hexa-nucleotide (1) while no penta-nucleotide was reported; *C. procera* the second highest was di-nucleotide (16), followed by tetra and hexa-nucleotide (8 each), while the least were tri-nucleotide and tri-nucleotide each with three repeats.

Long Repeats sequences in the four genomes of *D. velutina, G. siniacus, C. procera* and *G. sylvestre* Figure 5 generally reveals high number of palindromic and forward repeats. In *D. velutina* and *G. siniacus* forward repeats (21 and 25) are higher followed by palindromic (19 and 17), Reverse (11 and 9) and complements (3 and 1); While in *C. procera* and *G. sylvestre* forward (15 and 19) repeats are the majority followed by palindromic (16 and 23) followed by Reverse (11 and 9) then complement

Table 2. Gene assemblage in *D. velutina* and *G. siniacus* chloroplast genomes.

Category	Class of genes	Gene Identity
RNA genes	ribosomal RNA genes (rRNA)	*rrn5, rrn4.5, rrn16, rrn23*
	Transfer RNA (tRNA)	*trnH-GUG,trnK-UUU*[+], *trnQ-UUG, trnS-GCU, trnV-GAC*[a]*trnS-CGA*[+], *trnR-UCU,trnC-GC; trnD-GUC, trnY-GUA, trnE-UUC, trnT-GGU, trnS-UGA, trnfM-CAU, trnG-GCC, trnS-GGA, trnL-UAA*[+], *trnT-UGU, trnF-GAA, trnV-UAC;trnM-CAU, trnW-CCA, trnP-UGG, trnI-CAU*[a], *trnL-CAA*[a], *trnA-UGC*[+,a], *trnR-ACG*[a], *trnN-GUU*[a], *trnL-UAG*
Ribosome proteins	Small sub-unit of ribosome	*rps2, rps3, rps4, rps7*[a], *rps8, rps11, rps12*[a], *rps14, rps15,rps16*[+],*rps18, rps19*
Transcription genes	Large sub-unit of ribosome	*rpl2*[+,a], *rpl14, rpl16, rpl20, rpl22, rpl23*[a], *rpl32, rpl33, rpl36*
	DNA-dependent RNA polymerase	*rpoA, rpoB, rpoC1*[+], *rpoC2*
Protein genes	Photosystem I	*psaA, psaB, psaC, psaI, psaJ, ycf3*[++]
	Photosystem II	*psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbTpsbZ*
	Sub-unit of cytochrome	*petA, petB, petD, petG, petL, petN*
	Sub-unit of synthase	*atpA, atpB, atpE, atpF*[+], *atpH, atpI*
	Large sub-unit of rubisco	*rbcL*
	NADH dehydrogenase	*ndhA*[+], *ndhB*[+a], *ndhC, ndhD, ndhE, ndhF, ndhG, ndhH; ndhI; ndhJ;ndhK*
	ATP-dependent protease subunit P	*clpP*[++]
	Cp envelope membrane protein	*cemA*
Other genes	Maturase	*matK*
	Sub-unit acetyl-coA carboxylase	*accD*
	C-type cytochrome systhesis	*ccsA*
	Hypothetical proteins	*ycf2*[a], *ycf4*
	Component of TIC complex	*ycf1*[a]

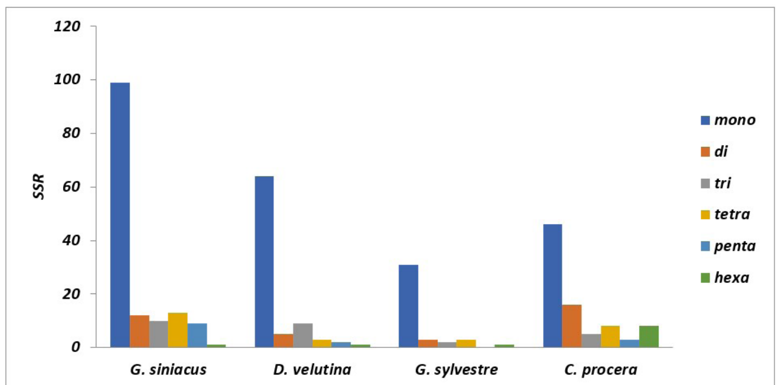

Figure 3. SSR types in *G. siniacus, D. velutina, G. sylvestre* and *C. procera* chloroplast genome sequence.

(1 and 3) being least in the two sequences (Figure 5). AT content of the *G. siniacus* is (62.1%-63.00%); while in *D. velutina* it was (61.55%-63.01%); these correspond with the hypothesis that all chloroplast genomes are hardly containing tandem (G) guanine or cytosine (C) but with only polyadenine (polyA) or polythymina (polyT) repeats. The SSRs were also called microsatellites.

3.3. Substitutions rates analyses

In the study of molecular evolution, the Ka/Ks ratio is used to explain the mechanism of DNA sequence evolution, for the reconstruction of phylogenies, and for the identification of protein-coding genes. It can be used as tool for estimation of the selective pressure of gene evolution, with a Ka/Ks ratio of >1 denoting positive selection and

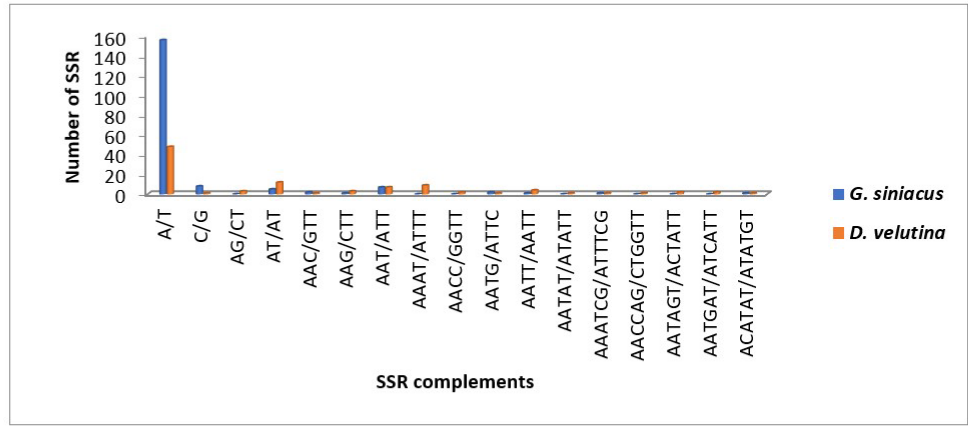

Figure 4. SSR complements in *G. siniacus* and *D. velutina* chloroplast genome sequence.

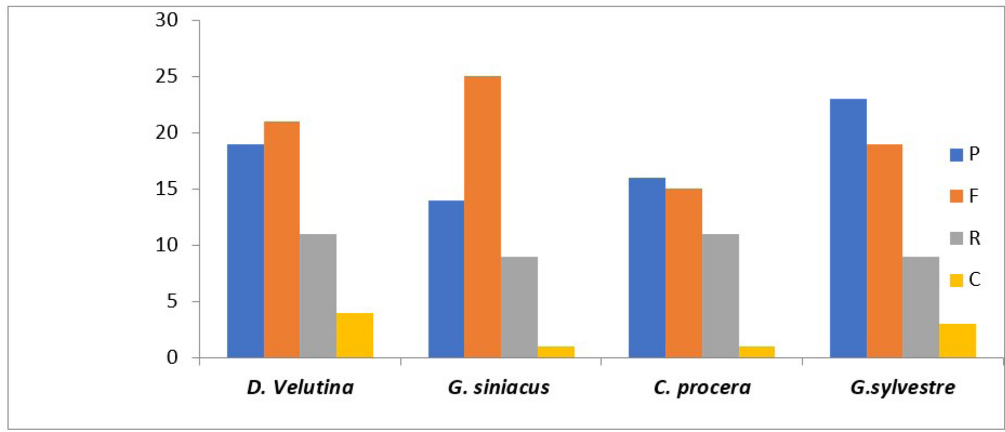

Figure 5. Long repeats sequences in *D. velutina, G. siniacus, C. procera* and *G. sylvestre* chloroplast genomes. P = palindromic; F = forward; R = reverse; and C = complement. The result of long repeats in Figure 5 indicate highest amount of forward long repeats (21), followed by palindromic (19).

a Ka/Ks ratio of <1 indicating negative selection; a value closer to 1 indicates neutral mutation.Synonymous (Ka) and non-synonymous (Ks) substitution rate and the Ka/Ks ration were determined to evaluate sequence divergence and relative selection in the protein coding genes. The result indicates low sequence divergence in most of the genes (Ks< 0.1) (Figure 6).

3.4. Chloroplast genome comparison of G. siniacus, D. velutina and G. sylvestre and C. procera

The whole chloroplast genomes of *G. siniacus*, *D. velutina* and *G. sylvestre* were compared and annotated *C. procera* was used as reference sequence (Figure 7) to reveal the features of variations. The output shows there is more variability at the IR regions than the LSC and SSC regions. The coding region is also conserved but non-coding region is less conserved. The most divergent regions are found at the *ycf2, psaB, ndhK, ndhB, rpl22, rpoc2, ycf15, petD* while at the coding region *matK, accD*. The Mvista comparison showed that the genomes were conserved with few variations noticed at the non-coding region; the genome of *G. siniacus* showed good candidacy for the identification and authentication of the taxa on the basis of its structural arrangement. These can be used as molecular markers for the identification of Asclepiadoideae Subfamily and Apocynaceae in general.

3.5. Inverted Repeats (IR) junction analysis

Calotropis procera, *Gymnema sylvestre, Gomphocarpus siniacus* and *Duvalia velutina* chloroplast genomes border junction comparison (Figure 8) indicates variations between three genes on the basis of their positions; genes such as *trnH-GUG*, *rps19* and *ycf1* were observed. *trnH-GUG* is located at the LSC-IRa border regions of *C. procera*, *Gymnema sylvestre, G. siniacus* and *D. velutina,* genomes while they varied in sizes (3 bp, 1bp, 16bp and 3bp); *rps19* and was located at the LSC region in *C. procera, G. sylvestre* and *G. siniacus* genomes, while in *D. velutina* it extended into IRb regions. The disparity due to contractions and expansion of the genome;*ycf1* is located at the extensions SSC-IRa border regions in *C. procera, G. sylvestre* and *D. velutina* thereby creating pseudo genes between the regions; while in *G. siniacus* its located at the SSC region.

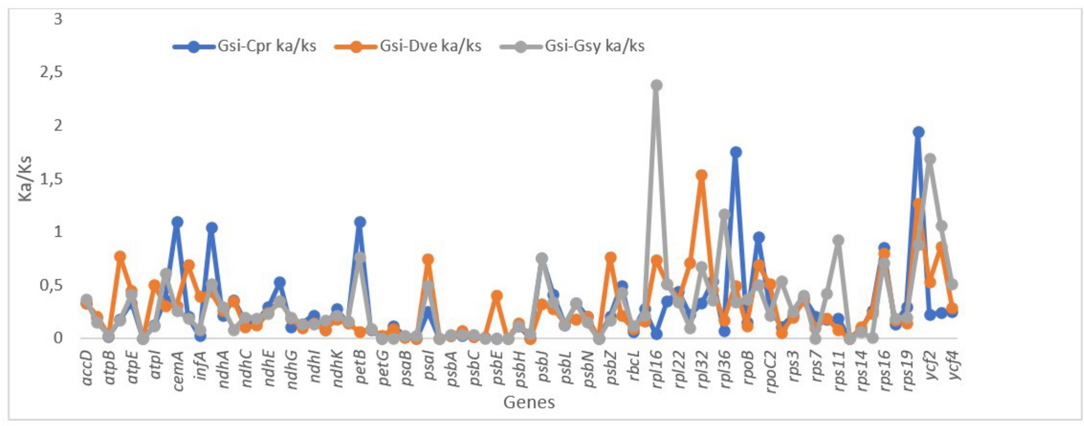

Figure 6. Synonymous Ka/ks ratio of *G. siniacus* against *C. procera, D. velutina* and *G. ylvestre*.

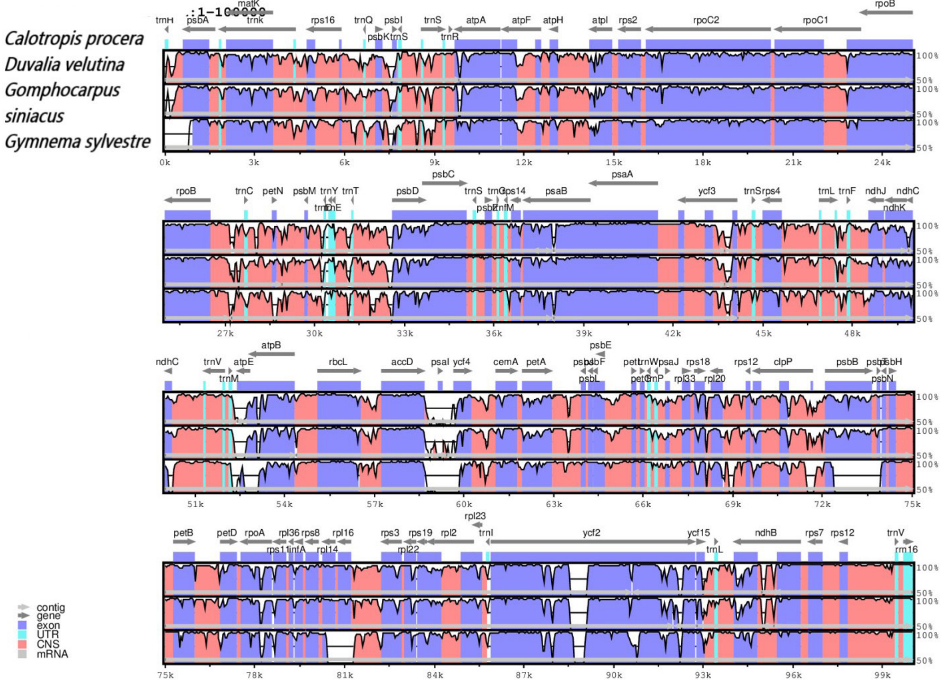

Figure 7. Sequence comparisons of four chloroplast genomes using mVISTA programe; with *C. procera* used as reference genomes.

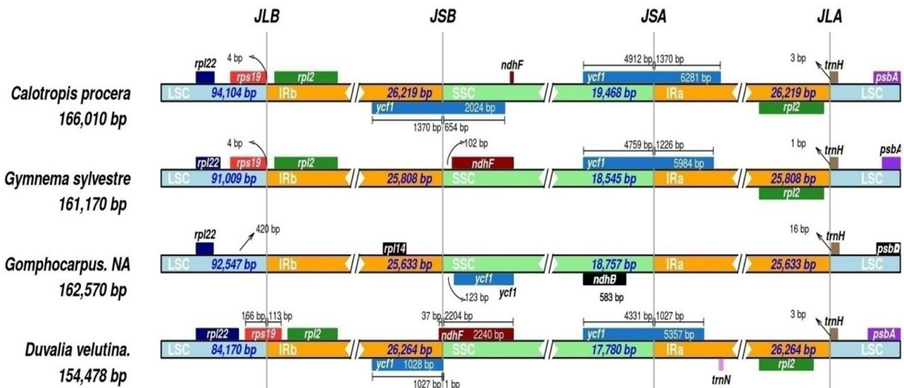

Figure 8. Comparative chloroplast sequences junctions of LSC, SSC and IR *in D. velutina, G. siniacus, and C. procera* and *G. sylvestre* genomes.

The phylogenetic tree (Figure 9) showed *Duvalia* and *Stapelia* are sister taxa and they should be regarded as separate tribes. The sister relationship between *Gomphocarpus* and *Calotropis* is also validated. *Gomphocarpus* and *Calotropis* were placed in the tribe Asclepiadea as sub tribe.

4. Discussion

Two species Asclepiadoideae were assembled and compared with two publicly available species where the sequence alignment, IR contraction and expansion were evaluated. We observed a pseudogenization of *ycf1* in *G. siniacus* sequence. Also the substitution rates were calculated. The four genomes compared were similar in some basic features such as gene contents, number of tRNA and rRNA genes, introns and GC contents. The similarity observed was due to the conserved nature of the chloroplast genome in angiosperm as reported by Ahmed et al. (2013), Li et al. (2019), Parks et al. (2009), Saina et al. (2018). Some species were also varied in their gene number as a result of loss or gain of an intron at either genus level or at family level (Menezes et al., 2018; Abdullah et al., 2019).

However, positioning of *rps19* at the IR region in the *G. siniacus* (Figure 8) was also observed by Cui et al. (2019) and Yaradua et al. (2019). Three genes were present in both genomes which is imperative to the findings in other species such as *Lycium babarum* (*Solanaceae*) and *Swertia mussotii* (*Gentianaceae*) where *rps19 andYcf1* were found to be pseudogenes (Cui et al., 2019; Xiang et al., 2016). This Serine-leucine (S–L) amino acid conversion happens in most angiosperms, as reported in *L. barbarum* and *L. chinense* (Cui et al., 2019) and also in *Dendrobium officinale* (Luo et al., 2014) as well as *Aristolochia debilis* (Zhou et al., 2017). . Ycf1 is located at the extensions of IRb and SSC regions there by creating pseudo genes between the regions. IR region is regarded as the most conserved regions in chloroplast genome (Cui et al., 2019; Zhou et al., 2017; Raubeson and Jansen, 2005).

Overall GC content of the genomes were 37.8% and 40.7% Figure 5 for *G. siniacus* and *D. velutina*, also the findings reveals high GC content at the IR region (35.2-33.2%) of the two genomes. This variation was also observed by Cui et al. (2019), Raveendar et al. (2015), Xiang et al. (2016), in which the authors attributed the variation to the localization of rRNA at IR region. The higher AT content at the third position in the coding was also observed by Cui et al. (2019), Xiang et al. (2016), He et al. (2017). This findings was used in the discrimination of chloroplast DNA from the Nuclear and mitochondrial DNA (Shen et al., 2018; Clegg et al., 1995).The GC contents of the cp genomes in this study is similar to the other cp genomes of Apocynaceae and its very much GC-lacking as a result it causes a very much bias towards A/T at the third codon positions (Qian et al., 2013). Mutations occur as a result of translation-preferred codons due to natural selection during evolution of the cp genomes (Yang et al., 2018) RNA editing is an evolutionary process that modify the genetic makeup of a genome by altering the precursor RNA's nucleotide sequence (Tsudzuki et al., 2001). This process of the post-transcriptional modification of precursor RNAs to alter their nucleotide sequences (Hoch et al., 1991). It sometimes occurs through the insertion and deletion of nucleotides, or specific nucleotide substitution (mostly C to U conversion) (Hoch et al., 1991). Since the first evidence of RNA editing was found in chloroplast in the *rpl2* transcript of maize (Freyer et al., 1997), it has been hunted out and systematically studied in the protein-coding transcripts of majority land plants lineages (Tillich et al., 2005), such as Arabidopsis thaliana (Tillich et al., 2010), *N. tabacum* (Yin et al., 2018), *Zea mays* (Maier et al., 1996), *Oryza sativa* (Corneille et al., 2000), *D. velutina* and *P. tomentosa* (Abba et al., 2020, 2021).

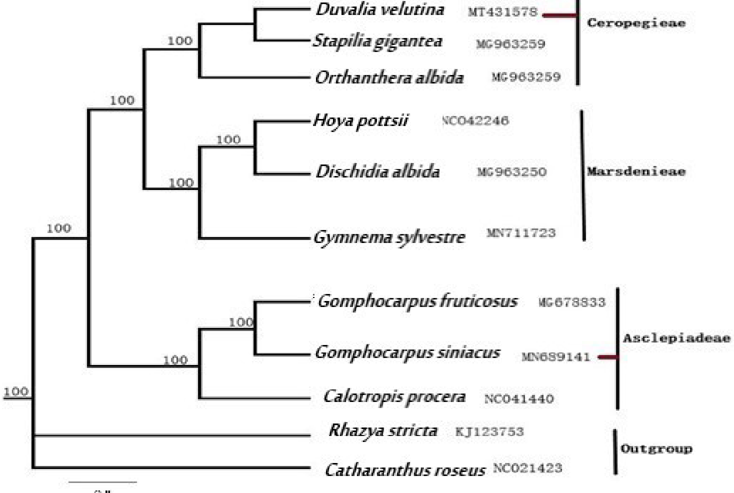

Figure 9. Phylogenetic tree of 11 taxa based on the complete chloroplast genomes using Bayesian Inference (BI) and Maximum Parsimony (MP) methods; which indicates the relationship within the eleven species of Apocynaceae. The numbers in the branch nodes represent Bootstrap Percentage (BP).

Most studies noted that start or stop codons were created by RNA editing which result in shortening of the size of translation products (Ozawa et al., 1997; Wakasugi et al., 1996; Yoshinaga et al., 1997). Also during production of new gene as a result of one striking case (Wakasugi et al., 1996); Our findings revealed that there is an codon initiation in *psbL* gene which is responsible for the production of PSII- L protein (Ozawa et al., 1997), as previously reported in tobacco (Bock et al., 1993; Kudla et al., 1992) and pepper (Kuntz et al., 1992) and spinach (Maier et al., 1996). RNA editing is common in cp genomes of angiosperms. It usually alters reading frames, mutation, as well as regulation of genes expressions of plants it however, serve as a corrective mechanism in the cp genomes of angiosperms.

RNA editing sites in the sequences of *G. siniacus* and *D. velutina* were high from Leucine to serine and mostly the codon conversion from hydrophilic to hydrophobic amino acids were also observed. This has also been reported in other angiosperms by Mehmood et al. (2020), Abdullah et al. (2019). The variation in the sizes of the genomes is as a resultof expansion and contraction of IR borders (Yang et al., 2016). This expansion was reported in *B. prionitis*, in Acanthaceae. The sizes of the inverted repeats were 25,104 bp in *D. velutina* and 25,461 bp in *G. siniacus.*

Four sequences were compared for IR borders, where three types of junctions were recognized based on the position of *rps19* gene, *trnH* and *ycf1-ndhF* positioning. In the first border there is similar orientation of the SSC, LSC and IRa and IRb in *D. velutina, C. procera* and *G. sylvestre.* In the *G. siniacus* sequence there is a clear variation in the orientations of Junctions. Secondly, uniform Border junction was observed in the four sequences while at the third position position a *ycf1* in *G. siniacus* was unique in its position because of its appearance at the forward strand while in *D. velutina, C. procera* and *G. sylvestre* it appeared in both forward and reverse strands.

Chloroplast genome has been reported to be much conserved in nature although there is report of variation between species as reported by Yang et al., 2016. The Mvista comparison showed that the genomes were conserved with few variations noticed at the non-coding region; the genome of *G. siniacus* showed good candidacy for the identification and authentication of the taxa on the basis of its structural arrangement (Rousseau-Gueutin et al., 2015; Yang et al., 2016). Alignment of four genomes shows variable regions in the four sequences such as *trnH-guG, rbcL, rps16-trnQ* and *rps19.* These can be used as molecular markers for the identification of Asclepiadoideae Subfamily and Apocynaceae in general.

We used the complete cp genomes to reconstitute a Phylogenetic tree and to establish the phylogenetic relationships, as well as tribal positions.The phylogenetic tree showed *Duvalia* and *Stapelia* are sister taxa as reported previously (Silva et al., 2012) therefore should be regarded as separate tribes. The sister relationship between *Gomphocarpus* and *Calotropis* is also validated. *Gomphocarpus* and *Calotropis* were placed in the tribe Asclepiadeae as sub tribe (Nazar et al., 2019; Sinha and Mondal, 2017). Recently Sinha and Mondal; Nazar et al., 2019 both classified *Gomphocarpus* under the tribe Asclepiadeae on the basis of molecular *trnL-trnF* markers. Our results indicate the position of *Duvalia* as member of the tribe Ceropegieae while *Gomphocarpus* has been placed under tribe Asclepiadeae.

5. Conclusion

The study involves sequencing and analysis of two species of Asclepiadoideae, *G. siniacus* and *D. velutina* (Apocynaceae). The structures of the two genomes were also compared in which different variable regions and SSR markers were unmasked. Also, the gene content arrangements and order were very much conserved. These detailed studies explain the evolutionary relationship among these two genomes which could help in identification, authentication, breeding and evolutionary studies of the family Apocynaceae.

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