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Comparative Chloroplast Genome Analysis and Evolutionary Relationships in Some Species of Asclepiadeae, Apocynaceae

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ABSTRACT

Background: Comparative study of the complete chloroplast genomes of some species in the Subtribe *Asclepiadeae* was conducted to evaluate the variations and similarities between the species and to resolve the Phylogenetic relationship within the subtribe. *P. tomentosa* has been used for medicinal uses in Saudi Arabia, Middle East, Africa and Brazil. It is used often in cosmetics and tanning industries, although it's very well utilized as a traditional medicine in many civilizations.

Methods: The genomes were compared using Mvista Bioinformatics tools to evaluate the inverted repeats (IR), large single copy (LSC) and small single copy (SSC) regions and also the border junctions were visualized with IR scope to express the expansion and contraction of the circular genome structure. While SSR markers were determined using the Reputer program, the genome map was done using OGDRAW (OrganellarGenomeDRAW).

Result: Observed variations of the Mvista alignments is mainly at the coding regions of the sequences, while IR borders were varied at the SSC region of *A. nivea* genome; with *ycf1* and *rps19* due to evolutionary events. The genome sizes of *C. procera* are 166,010 bp, *P. tomentosa* 164,213bp, *A. nivea* 161,592 bp and *C. wilfordii* 161,180 bp. GC contents of *A. nivea, C. wilfordii* and *P. tomentosa* are 38% respectively; while *C. procera* is the least with 37%; total SSR markers as well as the circular genome map were presented in this study.

Key words: Apocynaceae, Conservation, Molecular phylogeny, Phylogenomics, SSR Markers.

INTRODUCTION

Recent usage of the complete chloroplast genome in solving evolutionary questions has been on the rise due to its unique nature and its capability to unravel valuable information worthy of adopting, as well as the utilized widely in resolving phylogenetic problems. The nature of chloroplast varied as reported by different authors even though are similar in most angiosperms; in terms of their coding regions, sizes, tRNA genes and other related genes (Raubeson *et al.,* 2007; Daniell *et al.,* 2016; Yaradua *et al.,* 2019). Complete chloroplast genomes have been used as a vital tool in revealing information for phylogenetic reexamining of complex taxa; it is used vividly in resolving some unanswered questions in plant taxonomy recently (Ruhsam *et al.,* 2015). The chloroplast is a major distinctive feature of plants that is used in photosynthesis and production of starch, fatty acids, pigment and amino acid (Wicke *et al.,* 2011).

The majority of the land plant has a circular quadripartite shape with four different sizes; which is very well conserved in its genes contents and also partaking in translation, transcription and photosynthesis (Jansen *et al.,* 2005). Despite the conservative nature of the genes in cp genomes yet they undergo expansions and contractions over time which might result in loss and gains of introns which causes compounded rearrangements thereby causing variations in their contents and shapes from species to species. In addition to the evolutionary perspective, the chloroplast genome has important implications in chloroplast transformation such as the improved transgenic expression and absence of transgenic escape through pollen (Daniell *et al.,* 2016).

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P. tomentosa has been used for medicinal uses in Saudi Arabia, Middle East, Africa and Brazil. It is used often in cosmetics and tanning industries, although it's very well utilized as a traditional medicine in many civilizations, it is also used for the treatment of bronchitis, headache and tuberculosis among other uses. In the present study whole plastome genome sequence of *Pergularia tomentosa* was characterized and compared with previously reported cp genomes of *Calotropis procera, Asclepias nivea* and *Cynanchum wilfordii* from the Genbank database as such; the study is done to make comparative genomics analysis of complete chloroplast genome of *P. tomentosa, C. procera, Asclepias nivea* and *Cynanchum wilfordii* Asclepiadoideae, Apocynaceae and also to evaluate the evolution and characteristics of *accD* gene within the species.

MATERIALS AND METHODS

Plants sample collection and extractions of DNA

P. tomentosa plant material was collected from the Taif at Al-Shafa mountains (22° 4.9′31″ N. 40° 18.6′28″ E) a town about 25km from the holy city of Makkah and identified at the herbarium of KAAU Jeddah. Extraction of DNA was done according to the manual of the manufacture in which the leaves of *P. tomentosa* were washed with ethanol (70%) and Silica, followed by DNA extraction with DNeasy Plant Mini Kit (Qiagen, Inc., Germany). The quality of the DNA was checked using 1% agrose gel electrophoresis and Multiskan (Thermo Scientific Inc., USA). The DNA samples were sequenced at the Novogene, Beijing for paired-end reads 150 bp short reads and 350 bp size with Hiseq2500 (Illumina, USA).

Preparation of library, DNA sequencing and assembly

1.0 µg of the leaf sample was measured for DNA preparation to serve as an input. NEBNext DNA Library Kit was used to generate the sequencing libraries following the manufacturer's manual using the DNeasy Plant Mini Kit (Qiagen, Inc., Germany); with indices included in each sample. DNA was fragmented erratically to 350bp size by shearings; polished DNAs strand was then ligated with NBNext adapter for Illumina sequencing followed with P5, P7 indexing; also purification of PCR and later analyzed for the effective magnitude of the DNA by the Agilent 2100 and measured using RT-PCR. Raw reads obtained from the Illumina sequencers were filtered to obtain a (5 GB) with PRINSEQlite v0.20.4 (Schmieder and Edward, 2011). NOVOPlasty2.7.2 program (Dierckxsen *et al.,* 2016) was used for Assembly of the FastQ files with (Kmer = 32) to get the whole chloroplast genome out of the whole genome reads as well as the ndhAx1 to ndhAx2 ITS (intergenic spacer) of the *Cynanchum wilfordii (*KX352467K) were used as a seed while the sequence of *Calotropis procera* (NC_041440) as a reference. A complete chloroplast sequence was generated afterward.

Annotation of genes

Dual Organellar Genome Annotator program, (Wyman *et al.,* 2004) University of Texas was employed for annotation of *P. tomentosa* plastome in which manual adjustment of the stop and start codons were done; we then use tRNAscan-SE program www.lowaelab.ucsc.edu/tRNAscan-Se/in annotation of tRNAs.; while for drawing of chloroplast circular structure, an OGDRAW (Lohse *et al.,* 2007) was employed. The complete annotation was achieved and later deposited to GenBank with the assigned accession number (MN548766).

Repeats analysis

Simple sequence repeats (SSRs) were identified in the *P. tomentosa* chloroplast genome and genome of other three species of Asclepiadeae using the online software MIcroSAtellite (MISA) (Mayor *et al.,* 2000) with the following parameters: eight, five, four and three repeats units for mononucleotides, dinucleotides, trinucleotides and tetra, Penta, hexanucleotides SSR motifs respectively.

Long repeats identification

Reputer software (Kurtz *et al.,* 2001) was employed in tandem identifications, forward and a palindromic repeat of the genomes were identified following a manual method of 90% and >15 base pairs as sequence capture values.

RESULTS AND DISCUSSION

The result of the analysis which includes the complete chloroplast genomes features and map of *P. tomentosa* and comparison with *C. procera, Cynanchum wilfordii* and *Asclepias nivea* as well as the presentation of the evolution of *accD* gene in Asclepiadoideae*,* Apoceanaceae were discussed.

Genes with Introns

Table 1 is narrating features of genes with introns in *P. tomentosa* whole chloroplast genome with *clpP*, *accD* and *ycf3* both from LSC section of the genome were located at intron 2 and exon 3 respectively. The position of *accD and ycf3* gens agrees with the finding of Yaradua *et al.* (2019).

SSR Analysis

Table 2 shows the SSR markers in *P. tomentosa* with about 42 repeats sequences which are either palindromic or forward. The findings are similar to the studies by Huang *et al.,* 2020. The cp genome has a similar variability with the previously reported cp genomes of Asclepiadoideae subfamily such as the *C. wilfordii* (KT220733) (Park*, et al.,* 2018) and *C. procera* (NC041440). All the genomes had a quadripartite structure consisting of SSC, SSC regions and inverted repeats.

Table 1: Genes with introns of *P. tomentosa* chloroplast genome with lengh of exon and intron observed.

Gene	Position	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3
ycf1	SSC	552	953	538		
rps12	IR	235	545	09		
ndhB	IR	776	685	755		
rpl2	LSC	390	640	433		
trnK-UU	LSC	34	2489	36		
rps16	LSC	225	865	40		
trnG-UCC	LSC	22	672	36		
atpF	LSC	410	706	143		
rpoC1	LSC	1609	751	450		
clpP	LSC	383	682	293		
trnL-UAA	LSC	36	490	49		
accD	LSC	655	1194	194	627	06
ycf3	LSC	154	799	225	741	125
rpoC2	IR	433	640	390		
trnV-UAC	LSC	36	582	37		

Table 2: Total SSR Sequences in *P. tomentosa* genome.

Number	Repeat Size	Position 1	Type	Location 1	Position 2	Location 2	E-values
1	125	91252	F	*accD*	91273	*accD*	4.19E-66
2	108	163886	F	*ycf1*	163919	*ycf1*	7.20E-56
3	104	91252	F	*accD*	91294	*accD*	1.84E-53
4	103	89686	F	*accD*	89720	*accD*	7.37E-53
5	61	398	F	*ycf1*	452	*ycf1*	1.42E-27
6	61	398	P	*ycf1*	143713	*ycf1*	1.43E-27
7	98	163779	F	*IGS*	163919	*IGS*	7.55E-50
8	97	398	F	*ycf1*	434	*ycf1*	3.12E-49
9	97	398	P	*ycf1*	143713	*ycf1*	3.12E-49
10	97	416	P	*ycf1*	143731	*ycf1*	3.12E-49
11	97	143713	F	*ycf1*	143731	*ycf1*	3.02E-49
12	87	104044	F	*ycf1*	104086	*ycf1*	3.17E-43
13	83	91252	F	*accD*	91315	*accD*	8.11E-41
14	83	160417	F	*IGS*	91315	*IGS*	8.11E-41
15	79	398	F	*ycf1*	434	*ycf1*	2.18E-38
16	79	398	P	*ycf1*	143713	*ycf1*	2.18E-38
17	79	434	P	*ycf1*	143749	*ycf1*	2.18E-38
18	79	143713	F	*ndhB*	143749	*ndhB*	2.18E-38
19	76	91210	F	*IGS*	91378	*IGS*	1.33E-36
20	75	163886	F	*IGS*	163952	*IGS*	5.31E-36
21	71	18884	F	*ycf2*	18902	*ycf2*	1.37E-33
22	71	18884	P	*ycf2*	125253	*ycf2*	1.37E-33
23	71	18902	P	*ycf2*	125271	*ycf2*	1.37E-33
24	71	125253	F	*ycf2*	125271	*ycf2*	1.37E-33
25	69	89686	F	*accD*	89754	*accD*	2.80E-31
26	65	92238	F	*accD*	92292	*accD*	5.57E-29
27	65	113933	F	*infA*	113951	*infA*	5.57E-29
28	65	160072	F	*ycf1*	160090	*ycf1*	5.57E-29
29	65	163779	F	*IGS*	163845	*IGS*	5.57E-29
30	62	91252	F	*accD*	91336	*accD*	3.57E-28
31	61	398	F	*ycf1*	452	*ycf1*	1.43E-27
34	61	143713	F	*ycf1*	143767	*ycf1*	1.43E-27
35	53	18884	F	*ycf2*	18920	*ycf2*	9.35E-23
36	53	18884	P	*ycf2*	125253	*ycf2*	9.35E-23
37	53	18920	P	*ycf2*	125289	*ycf2*	9.35E-23
38	53	125253	F	*ycf2*	125289	*ycf2*	9.35E-23
39	50	144226	P	*ycf1*	164163	*ycf1*	5.98E-21
40	50	160212	F	*ycf1*	160230	*ycf1*	5.98E-21
41	48	92150	F	*accD*	92234	*accD*	9.57E-20
42	47	113933	F	*infA*	113969	*infA*	3.83E-19
43	47	160072	F	*ycf1*	160108	*ycf1*	3.83E-19
44	45	90522	F	*accD*	91200	*accD*	6.13E-18

Genome features analysis of *P. tomentosa, C. procera, C. wilfordii* and *A. nivea*

Table 3 Indicates genome features of *P. tomentosa, C. procera, C.wilfordii* and *A. nivea,* in which the *C. procera* genome size is 166,010 bp, *P. tomentosa* 164,213 bp, *A. nivea* 161,592 bp and *C. wilfordii* 161,180 bp (Fig 1). The total number of genes was highest in *C. wilfordii* 168, *C. procera* 163, while *P. tomentosa* and *A. nivea* are the least number of genes with 161 each However, the GC content of the genomes showed highest *in A. nivea, C. wilfordii* and *P. tomentosa* with 38% while *C. procera* is the least with 37%. tRNA content is highest in *A. nivea* and *P. tomentosa* 46% and low in *C. procera* and *C. wilfordii* 42% each; total rRNA genes were same in all the plants 8 each; the size of the inverted repeats (IR) were highest in *C. procera* 26,219bp, followed by A. *nivea* 25,472bp, *C. wilfordii* 24,637bp and *P. tomentosa* 21411bp. For the size of large singe copy (LSC) *C. procera* is the highest 94,104bp, then

Table 3: Genome features of *Pergularia tomentosa, Calotropis procera, Cynanchum wilfordii and Asclepias nivea.*

Plant ID	Genome size (bp)	Overall gene	GenBank ID	GC content (%)	Protein coding gene	tRNA gene	rRNA gene	IR (bp)	LSC (bp)	SSC (bp)
Perguleria tomentosa	164213bp	161	MN548766	38	108	46	8	21411	80102	17022
Calotropis procera	166010bp	163	NC_041440	37	108	42	8	26219	94104	19468
Cynanchum wilfordii	161180bp	168	KX352467K	38	108	42	8	24637	91976	19930
Asclepias nivea	161592bp	161	KF539844	38	108	46	8	25472	91971	18771

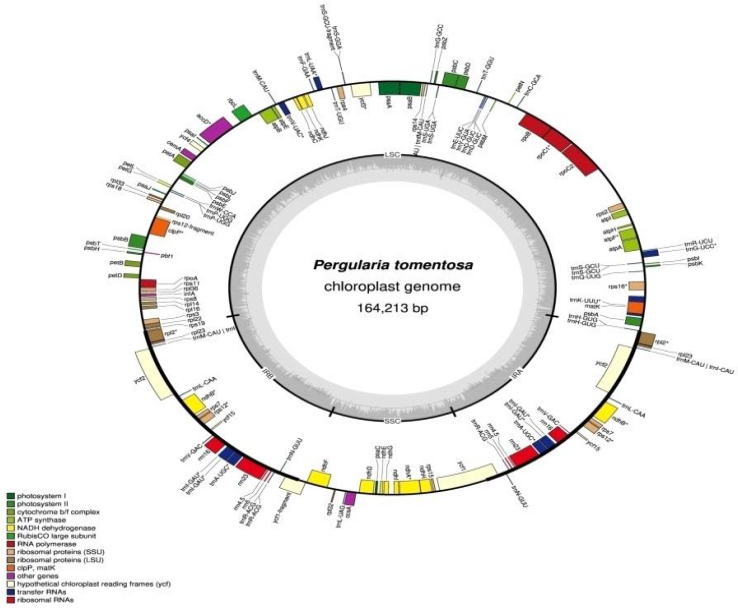


Fig 1: Circular Complete Chloroplast genomes of *P. tomentosa* with genes which are residing within the circular shape are on clockwise direction while those outside are at the anti-clockwise.

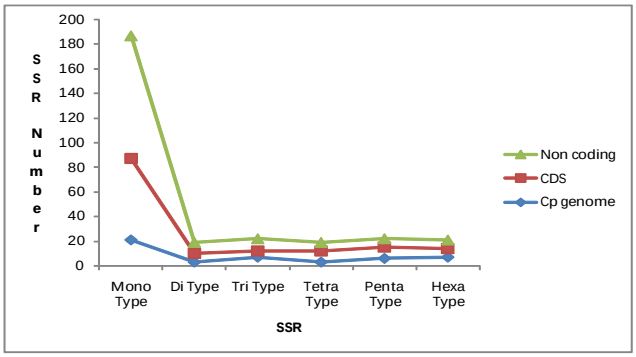


Fig 2: Features of the non-protein genes, SSR types and coding genes in *P. tomentosa* genome.

C. wilfordii 91,976bp, *A. niveae* 91,971bp and *P. tomentosa* being the least with 80,102bp; Also the size of Small single copy (SSC) of the plants *C. wilfordii* has the highest size with 19,930bp, then *C. procera* 19,468bp, followed by *A. niveae* with 18,771 and *P. tomentosa* with 17,022bp.

Comparative genome analysis using Mvista alignment

Comparative genomic analysis in Fig 3 indicate that genes at non-coding regions are more conserved compared to the ones at the coding regions; such as *ycf15*, *cemA*, *rpl16* and *clpP* genes were the most divergent regions observed. Genetic diversity in angiosperm chloroplast genomes has been reported in families such as the Angiosperms such as the Amaranthaceae, Caryophyllaceae, with some showing a character of loss of introns in coding regions (Daniell *et al.,* 2008). The nature of chloroplast varied as reported by different authors even though are also similar in most angiosperms; in terms of the coding regions sizes, complete gene number, complete tRNA genes and complete protein genes (Raubeson and Jansen, 2005; Daniell *et al.,* 2016; Yaradua *et al.,* 2019). Distribution in the number of SSR Fig 2. revealed that non-coding region has higher SSR, followed by the coding region and the cp genome region.

Furthermore, the *accD* gene deals with the formation of fatty acids in plants programming the betacarboxyl transferase a derivative of acetyl coA (Sasaki and Nagano, 2004; Rousseau-Guetin *et al.,* 2013; Kode *et al.,* 2005). It was also reported that fatty acids contents and metabolic activities in plants are influenced by the *accD* gene expression (Lee *et al.,* 2004; Nakkew *et al.,* 2008); *accD* gene is a pseudo-gene in some angiosperm (Xiang *et al.,* 2016) while exist as a complete gene in others (Ni *et al.,* 2016b) the result of these study indicate about 6 highly conserved regions in the *accD* of *P. tomentosa* and *A. nivea* while two and four highly conserved regions detected on *accD* gene of *C. wilfordii* and *C. procera*, respectively.

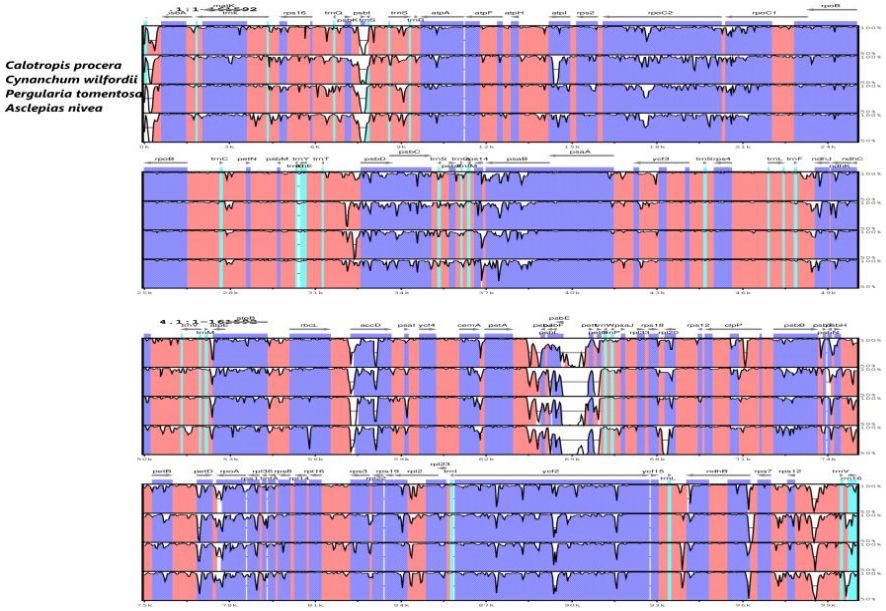


Fig 3: Sequence alignment of four complete chloroplast of sub family *Asclepiadoideae* in *Apoceanacee* using mVISTA program. Blue colouration in the figure indicates protien coding regions, top arrow denotes the transcription axis, pink colour represent the section of conserved noncoding sequences CNS while the fair green represent total tRNA and rRNA in the sequences.

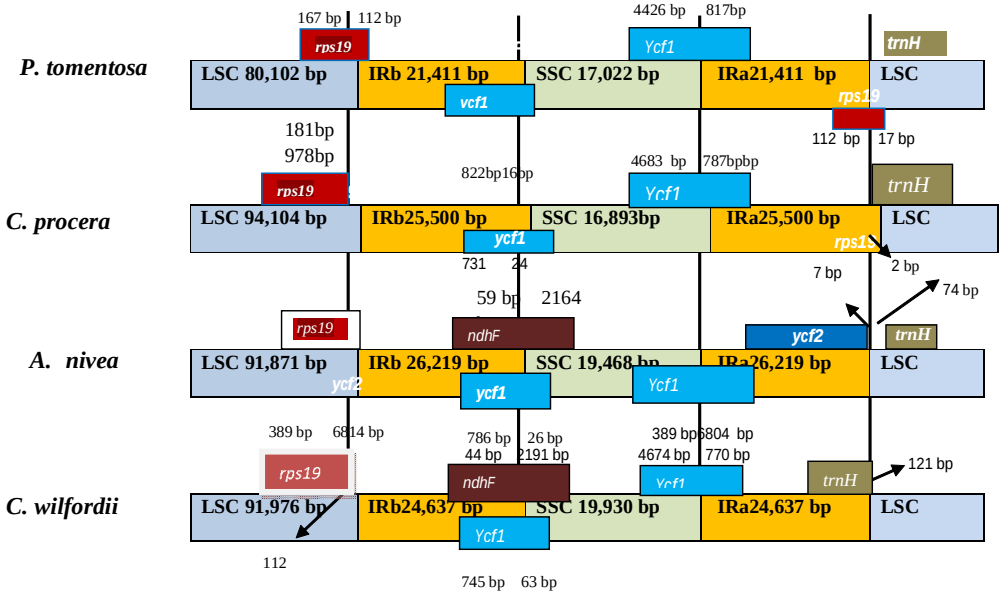


Fig 4: Genome borders comparison of IR, SSC and LSC of four species of Asclepideae.

IR border junction analysis

Variations exist between the chloroplast genomes of *P. tomentosa. C. procera, A. nivea* and *C. wilfordii* as seen in Fig 4 where *trnH*, *rpl22*, *ycf1*, *ndhF* and *rps19* genes are designated by the junction of IR andSSC regions. Variation was also observed at the SSC section of *A. nivea* genome; with *ycf1* and *rps19*. Angiosperms chloroplast genome was generally known for its uniqueness and conservative features in structures and topology; even with many evolutionary events there exists slight changes that lead to the variations among members of the same taxonomic groups due to contraction in the borders of the genomes. The comparison between plastome genomes of *P. tomentosa, C. procera, A. nivea* and *C. wilfordii* reveals some variations in their genome architecture.

Phylogenetic analysis

Phylogenetic inference revealed that the genus *Pergularia* is a monophyletic genus (Fig 5) and a sister genus to *Calotropis* and *Asclepias*. This result agrees with the findings of Rapini *et al.,* 2012 and Abba *et al.,* 2020 based on

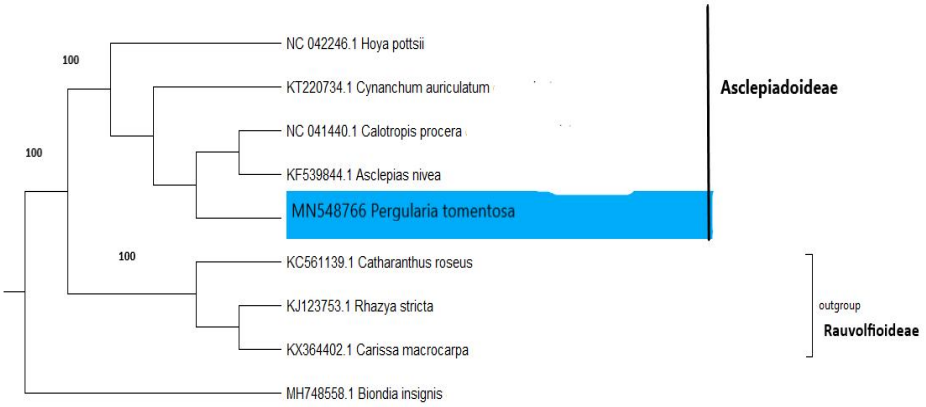


Fig 5: Phylogenetic tree of Asclepiadoideae subfamily using complete Chloroplast genomes to infer Bayesian inference of the 9 species from Apocynaceae family.

complete chloroplast genome sequences of some species in the subfamily Asclepiadoideae. Therefore, this study highlighted the chloroplast genome architecture in Asclepiadoideae and the variations among the species studied. There is a close resemblance in the genome's features; identified SSR markers (Fig 2) could be used in population genetics and Phylogenetic studies of the subfamily Asclepiadoideae.

CONCLUSION

The study involving the comparative genome sequence analysis of *P. tomentosa* and other species of Asclepideae was done in which the genome features such as the SSR markers and *accD* gene evolution were evaluated; as such that the information could be used for future decision making in Asclepiadoideae as well as the studies of evolutionary relationship, herb-genomics, plant breeding and conservation.

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