A Repertory of Rearrangements and the Loss of an Inverted Repeat Region in *Passiflora* Chloroplast Genomes

Luiz Augusto Cauz-Santos¹, Zirlane Portugal da Costa¹, Caroline Callot², Stéphane Cauet², Maria Imaculada Zucchini³, Hélène Bergès², T., Cássio van den Berg¹, ⁴, and Maria Lucia Carneiro Vieira ¹, ², ³, ⁴.

¹Departamento de Genética, Escola Superior de Agricultura “Luiz de Queiroz,” Universidade de São Paulo, Piracicaba, SP, Brazil
²Centre National de Ressources Génomiques Végétales, INRA, Auzeville, Castanet-Tolosan, France
³Polo Regional de Desenvolvimento Tecnológico do Centro Sul, Agência Paulista de Tecnologia dos Agronegócios, Piracicaba, SP, Brazil
⁴Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, BA, Brazil

†Corresponding author: E-mail: mlcvieir@usp.br.
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²Present address: INARI Agriculture, Cambridge, MA

Abstract

Chloroplast genomes (cpDNA) in angiosperms are usually highly conserved. Although rearrangements have been observed in some lineages, such as *Passiflora*, the mechanisms that lead to rearrangements are still poorly elucidated. In the present study, we obtained 20 new chloroplast genomes (18 species from the genus *Passiflora*, and *Dilkea retusa* and *Mitostemma brevifilis* from the family *Passifloraceae*) in order to investigate cpDNA evolutionary history in this group. *Passiflora* cpDNAs vary in size considerably, with ~50 kb between shortest and longest. Large inverted repeat (IR) expansions were identified, and at the extreme opposite, the loss of an IR was detected for the first time in *Passiflora*, a rare event in angiosperms. The loss of an IR region was detected in *Passiflora capsularis* and *Passiflora costaricensis*, a species in which occasional biparental chloroplast inheritance has previously been reported.

A repertory of rearrangements such as inversions and gene losses were detected, making *Passiflora* one of the few groups with complex chloroplast genome evolution. We also performed a phylogenomic study based on all the available cp genomes and our analysis implies that there is a need to reconsider the taxonomic classifications of some species in the group.

Key words: chloroplast genome (plastome) rearrangements, *Passiflora*, inverted repeat (IR) loss, *Passiflora* classification.

Introduction

In higher plants, most chloroplast (cp) genomes have a quadripartite structure consisting of two copies of inverted repeats (IRs) separating two single copy regions, a large (LSC) and a small (SSC) region (Sugiura 1992; Yang et al. 2010). Although highly conserved in their structural organization, cp genomes may exhibit deletions, including gene loss, and rearrangements, such as IR inversions and expansions or retractions.
These rearrangements are well documented, for instance, in *Hevea brasiliensis* (Euphorbiaceae) (Tangphatsornruang et al. 2011) and in species of riverweeds (Podostemaceae) (Bedoya et al. 2019), both families belonging to the Malpighiales. Some other plant families, such as Fabaceae (Cai et al. 2011) and in species of riverweeds (Podostemaceae) (Bedoya et al. 2011), both families belonging to the Malpighiales. Some other plant families, such as Fabaceae (Cai et al. 2011) and in species of riverweeds (Podostemaceae) (Bedoya et al. 2011), both families belonging to the Malpighiales.

However, the mechanisms which lead to rearrangements in plastid genomes (plastomes) are still poorly elucidated. Among these mechanisms, intramolecular homologous recombination mediated by the presence of repeat structures at the boundaries of the rearranged region plays a role in bringing about structural changes (Ogihara et al. 1988; Wu, et al. 1981; Chumley et al. 2006; Weng et al. 2014). In addition, foreign DNA insertions (large open reading frames, for instance) have led to extensive cp genome rearrangements in Campanulaceae (Haberle et al. 2008), Pinaceae and other conifers (Wu, Lin, et al. 2011; Wu, Wang, et al. 2011), and Geraniaceae (Guisinger et al. 2011; Weng et al. 2014), also exhibit a high number of rearrangements in their cp genomes.

In our study, we sequenced, assembled, and annotated 18 chloroplast genomes representing the four subgenera in the genus *Passiflora*, along with the Passifloraceae *Dilkea retusa* and *Mitostemma brevifilis*, and extensive sequence rearrangements were found. We were able to address the following questions: Are the frequency and type of cpDNA sequence rearrangements particular to each subgenus? And what is the significance of these rearrangements for *Passiflora* intrageneric classification?

**Materials and Methods**

**Plant Material**

In total, 18 species of the genus *Passiflora* were analyzed, belonging to the four subgenera: *Astrophaea* (3), *Decaloba* (5), *Deidamioides* (2), and *Passiflora* (8). Of these species, 17 were field collected, and *Passiflora costaricensis* (*Decaloba*), a species native to Central America, was obtained from the Italian National Collection of *Passiflora*. In addition, two species of *Passifloraceae* (*D. retusa* and *M. brevifilis*) were also field collected and comparative studies conducted (supplementary table S1, Supplementary Material online). All plant accessions are registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN, Brazil).

**Intact Chloroplast Isolation by Sucrose Gradient Centrifugation and cpDNA Extraction**

Intact chloroplast organelles were isolated using the sucrose gradient method (Takamatsu et al. 2018). Some 20 g of fresh leaves from each plant were frozen in liquid nitrogen and macerated. The material was resuspended in 200 ml of isolation buffer (50 mM Tris–HCl [pH 8.0], 0.35 M sucrose, 7 mM ethylenediaminetetraacetic acid, 5 mM 2-mercaptoethanol, and 0.1% bovine serum albumin) and incubated for 10 min.
in the dark. The suspension was filtered through two layers of gauze and then two layers of Miracloth (Merck), and the filtrate centrifuged at 1,000 × g for 10 min. Finally, the pellet was washed in 50 ml of isolation buffer and centrifuged at 1,000 × g for 10 min.

The pellet was resuspended in 5 ml of isolation buffer and the suspension slowly laid out in a 20/45% sucrose density batch gradient in 50 mM Tris–HCl (pH 8.0), 0.3 M sorbitol, and ethylenediaminetetraacetic acid 7 mM. The gradient was centrifuged at 2,000 × g for 30 min. The green band formed at the interface containing the intact chloroplasts was collected, diluted with three volumes of isolation buffer, and centrifuged at 3,000 × g for 10 min to obtain the purified chloroplasts in the pellet.

The pellet was resuspended in 2% CTAB buffer to promote lysis. The suspension was then incubated at 65 °C for 1 h with stirring. The supernatant was extracted 2× with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 × g for 20 min. An equal volume of isopropanol was added to the upper layer and incubated at 20 °C for 1 h. Finally, the aqueous phase was centrifuged at 10,000 × g for 20 min, and the cpDNA pellet washed with ethanol (70%), dried, and resuspended in 40 μl of Tris–ethylenediaminetetraacetic acid buffer.

Chloroplast Genome Sequencing and Assembly

Illumina sequencing libraries were constructed using a total of 100 ng of input cpDNA and the Nextera DNA Flex library Kit, following the manufacturer’s instructions. The sequencing was performed on the Illumina NextSeq platform (Fundação Hemonceto de Ribeirão Preto, Brazil) in two distinct runs, the first containing six species using paired-end sequencing (2 × 76 bp), and the second 13 species using paired-end sequencing (2× 150 bp) (supplementary table S2, Supplementary Material online). The paired-end reads were initially trimmed and filtered in Trimmmomatic v. 0.39 (Bolger et al. 2014). Adapter trimming was performed based on ILLUMINAclip:NexteraPE-PE.fa:2:30:10:2, and using the following quality filtering parameters: sliding window of 10:20, leading of 20, trailing of 20, and minimum length of 36 bp.

The filtered reads were de novo assembled in NOVOPlasty v. 3.8.3 (Dierckxsens et al. 2017), using the protein-coding genes psbA, rbcL, and rpoB from the P. edulis cp genome (NC_034285.1) as seeds. The k-mer sizes used in the assembly varied from 23 to 39 (supplementary table S2, Supplementary Material online). In order to obtain the final assembly, and taking into account that there were overlapping regions, the cp sequences were merged in Geneious v. 2019.1.2 (https://www.geneious.com, last accessed April 2020) using the “de novo Assembly” function. Finally, the correctness of the assembly was checked, and coverage estimated using the “Map to reference” function to map the paired-end raw reads onto the final assembled cp genome.

The plastid DNA sequence of P. costaricensis was obtained by long-read sequencing. Barcoded, large-insert (10 kb) libraries were constructed using 150 ng of pure high-molecular-weight DNA. Sequencing was performed in an SMRT cell using P6 polymerase with C4 chemistry on the PacBio RSII instrument at the Uppsala NGI Platform (Uppsala University, Sweden). The data were filtered to obtain high-quality reads (reads with quality < 0.75 and length < 500 bp were removed). The sequences were assembled using the CANU assembler with default parameters (Koren et al. 2017). Subsequently, the chloroplast contigs were extracted in Geneious by mapping the resulting contigs to the Passiflora complete cp genomes obtained in this study. A final assembled cp genome of P. costaricensis was obtained in Geneious by joining the contigs using the “de novo Assembly” function (supplementary table S2, Supplementary Material online). Finally, the raw reads were mapped onto the final contig using the “Map to reference” function.

Primer design and polymerase chain reaction (PCR) were applied to confirm the positioning of some distinct cpDNA arrangements (supplementary table S3, Supplementary Material online). Amplification reactions were performed using 20 ng template DNA, 1× buffer, 1 mM MgCl₂, 0.2 mM of each dNTP, 0.3 μM of the forward and reverse primers, 1.2 U Go Taq Flex DNA polymerase (Promega, Madison, WI), and ultra-pure water added to bring the final volume up to 20 μl. The amplification program was as follows: 95 °C for 5 min, 35 cycles at 95 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min, followed by a final 8 min of incubation at 72 °C. The amplified fragments were checked on 1% (w/v) agarose gel with a 1,000-bp molecular standard Invitrogen ladder (Carlsbad, CA).

Chloroplast Genome Annotation, Identification of Repeated Elements, and Comparative Analysis

The annotation of cp genomes was carried out in the GeSeq (Organellar Genome Annotation) online program (Tillich et al. 2017) with default settings to identify protein-coding gene sequences, rRNAs, and tRNAs based on the chloroplast reference sequences and BLAST homology searches, followed by manual corrections for start and stop codons and intron positions in GenomeView software. All tRNA genes were further confirmed using the tRNAscan-SE and ARAGORN online search server (Laslett and Canback 2004; Lowe and Chan 2016). Pseudogenes were classified based on nucleotide losses in sequences or the presence of internal stop codons. OGRDRAW software was used for constructing the circular cp genome map (Greiner et al. 2019).

REPuter software (Kurtz et al. 2001) was used to identify direct and palindromic repeat elements based on the following criteria: minimum repeat size ≥30 bp and sequence identities ≥90% (Hamming distance equal to 3).
Two different multiple sequence alignments were run in Progressive Mauve v.2.4.0 (Darling et al. 2010) to identify possible rearrangements in the cpDNA molecules. The first contained 11 cp genomes to represent the diversity in size and structure in each Passiflora subgenus, in addition to the cp genomes of *D. retusa* and *M. brevifilis* (Passifloraceae), with *Populus trichocarpa* (Salicaceae) as a reference. The second alignment contained all 20 cp genomes studied herein. Subsequently, to detect possible expansions or contractions in the IR regions, the IR boundaries of both LSC and SSC regions with full annotations for the adjacent genes were analyzed across the cp molecules used in the first multiple sequence alignment. In addition to reconstructing the evolutionary history of *Passiflora* chloroplast genomes, we analyzed the plastome structures run in the first multiple sequence alignment against the available GenBank cp genomes of *Passiflora foetida*, *Passiflora tetrandra*, *Passiflora obovata*, *Passiflora auriculate*, and *Passiflora biflora*, studied by Shrestha et al. (2019) (supplementary table S4, Supplementary Material online).

### Phylogenomic Studies

We performed a phylogenomic study based on all the available cp genomes of passionflowers (49 species in total, 20 generated in this study and 29 species whose cp DNA sequences were imported from the GenBank database, supplementary table S4, Supplementary Material online).

A set of 68 passionflower chloroplast protein-coding genes was used in a phylogenetic analysis, and the species *Po. trichocarpa* (Salicaceae) used as the outgroup, with the aim of obtaining a rooted tree (supplementary table S5, Supplementary Material online).

A local python pipeline was used to extract each gene in the data set (consisting of 50 taxa), align them individually at nucleotide level in MUSCLE and make an interleaved Nexus matrix consisting of all individual alignments. The resulting matrix was then analyzed in ModelFinder (Kalyaanamoorthy et al. 2017) to determine the best partition scheme and evolutionary models in accordance with the Akaike information criterion (AIC), and treating each gene alignment as a charset. Because the partition scheme indicated by ModelFinder is an a priori test based on point estimates, and a single model was selected with GTR + G + I for the whole data set, we decided to include an alternative model with partition based on codon position with separate parameters in GTR + G + I for each codon position, and then subsequently test the two-partition scheme using Bayes Factors. In both partitioned and nonpartitioned analyses, the Markov chain Monte Carlo algorithm was run in MrBayes (Ronquist et al. 2012) for 10,000,000 generations, sampling one tree every 1,000 generations and discarding the first 25% of trees as burn-in, to estimate the values of posterior probabilities (PPs). The convergence of the runs was monitored based on an average standard deviation of split frequencies below 0.01, potential scale reduction factor values close to 1.0, and ESS values above 1,000. Finally, the phylogenetic trees were visualized using FigTree version 1.4.4.

### Results

#### Chloroplast Genome Features in Passifloraceae

In *Passiflora*, a large variation in cpDNA sequence length was observed (~55 kb), ranging from 113,114 bp in *Passiflora capsularis* (subgenus Decaloba) to 167,953 bp in *Passiflora deidamioides* (subgenus Deidamioides) (supplementary fig. S1, Supplementary Material online, and table 1). The majority had the typical quadripartite structure; the length of the LSC region ranged from 57,244 bp in *Passiflora suberosa* (subgenus Decaloba) to 90,064 bp in *Passiflora rhamnifolia* (subgenus Astrophea), whereas the SSC region varied from 12,854 in *Passiflora cerradensis* (subgenus Astrophea) to 13,744 bp in *P. deidamioides*. IRs ranged from 21,928 in *Passiflora edmundoi* (subgenus Passiflora) to 43,626 bp in *P. suberosa* (subgenus Decaloba). The cpDNA sequence lengths of the Passifloraceae *D. retusa* and *M. brevifilis* were similar to those of the Astrophea species, which had the shortest SSC region sequenced in this study. The GC content of all 20 cp genomes was very similar (average 36.7%).

Comparing the four subgenera, *Decaloba* was the subgenus with the highest variation, ranging from 113,114 bp in *P. capsularis* and 114,230 in *P. costaricensis*, the smallest cp genomes sequenced herein due to the loss of an IR region (fig. 1), up to 158,313 bp in *P. suberosa*. This loss of the IR region in *P. capsularis* and *P. costaricensis*, particularly the IRa, was confirmed by mapping the raw reads onto the assembled genomes that resulted in continuity of coverage along the cp genome molecule. In addition, the IR loss was confirmed by PCR (supplementary table S3, Supplementary Material online). In contrast, the largest cp genomes were observed for the two species of the *Deidamioides* subgenus. The *Astrophea* subgenus also had large cpDNA molecules, whereas in the eight species of the *Passiflora* subgenus (which contains the vast majority of the species described in the genus), the cpDNA ranged from 142,737 bp (*P. edmundoi*) to 151,920 bp (*Passiflora miniata*).

The cp genomes contained between 102 and 109 unique genes, and this variation is related to the protein-coding genes identified when species were compared (68–75 protein-coding genes), because all species were found to have the same tRNA (30) and rRNA (4) gene content. Chloroplast genes are involved in different biological processes and were annotated accordingly with functional categories. Due to duplication and the emergence of IR regions during the evolutionary history of the genus, 18–38 genes were found to have two copies, including protein-coding genes, tRNAs, and all four rRNAs. The total number of genes ranged from 102...
| Species                      | Taxonomic Classification Subgenus/ Supersections, Sections, or Series | cp Genome Size (bp) | LSC (bp) | SSC (bp) | IR (bp) | Total GC % | No. Unique Genes | Protein-Coding Genes | tRNAs | rRNAs | NCBI Accession No. |
|-----------------------------|--------------------------------------------------------------------------|----------------------|----------|----------|---------|-------------|-------------------|----------------------|-------|-------|-------------------|
| Passiflora cerradensis      | Subgenus Astrophea/section Capreolata                               | 164,515              | 84,143   | 12,854   | 33,759  | 36.6        | 107               | 73                  | 30    | 4     | MT525871           |
| Passiflora haematostigma    | Subgenus Astrophea/section                                           | 163,775              | 89,717   | 12,876   | 30,591  | 36.5        | 106               | 72                  | 30    | 4     | MT525875           |
| Passiflora rhamnifolia      | Subgenus Astrophea/section                                           | 162,217              | 90,064   | 12,921   | 29,616  | 36.4        | 106               | 72                  | 30    | 4     | MT525882           |
| Passiflora candollei        | Subgenus Decaloba/section Decaloba                                   | 138,081              | 72,565   | 13,506   | 26,005  | 37.2        | 104               | 70                  | 30    | 4     | MT525870           |
| Passiflora capsularis       | Subgenus Decaloba/section Xerogona                                   | 113,114              | a        | a        | a       | 36.1        | 102               | 68                  | 30    | 4     | MT525883           |
| Passiflora costaricensis    | Subgenus Decaloba/section Xerogona                                   | 114,230              | a        | a        | a       | 36.1        | 102               | 68                  | 30    | 4     | MT473979           |
| Passiflora suberosa         | Subgenus Decaloba/section Geca                                      | 158,313              | 57,244   | 13,817   | 43,626  | 37.2        | 103               | 69                  | 30    | 4     | MT525868           |
| Passiflora vespertilio      | Subgenus Decaloba/section Decaloba                                   | 138,456              | 72,902   | 13,158   | 26,196  | 37.1        | 104               | 70                  | 30    | 4     | MT525880           |
| Passiflora contracta        | Subgenus Deidamioides/section Tetrastylis                            | 166,558              | 87,313   | 13,513   | 32,866  | 36.7        | 107               | 73                  | 30    | 4     | MT533196           |
| Passiflora deidamioides     | Subgenus Deidamioides/section                                       | 167,953              | 82,571   | 13,744   | 35,819  | 36.8        | 108               | 74                  | 30    | 4     | MT525873           |
| Passiflora alata            | Subgenus Passiflorasupersection Laurifolia/ section Quadrangularis  | 147,773              | 85,535   | 13,494   | 24,372  | 36.9        | 105               | 71                  | 30    | 4     | MT525869           |
| Passiflora cristalina       | Subgenus Passiflorasupersection Distephana                            | 145,054              | 85,662   | 13,530   | 22,931  | 36.9        | 104               | 70                  | 30    | 4     | MT525872           |
| Passiflora edmundoi         | Subgenus Passiflorasupersection Stipulata/ section Kermesinae        | 142,737              | 85,567   | 13,314   | 21,928  | 37.2        | 105               | 71                  | 30    | 4     | MT525874           |
| Passiflora loefgrenii       | Subgenus Passiflorasupersection Stipulata/ section Kermesinae        | 146,537              | 86,370   | 13,267   | 23,450  | 37.1        | 104               | 70                  | 30    | 4     | MT525876           |
| Passiflora miniata          | Subgenus Passiflorasupersection Distephana                            | 151,920              | 85,863   | 13,477   | 26,290  | 37.0        | 105               | 71                  | 30    | 4     | MT525877           |
| Passiflora mucronata        | Subgenus Passiflorasupersection Stipulata/ section Granadillastum    | 150,839              | 84,839   | 13,552   | 26,224  | 36.9        | 104               | 70                  | 30    | 4     | MT525878           |
| Passiflora recurva          | Subgenus Passiflorasupersection Passiflor/ series Passiflor           | 151,837              | 85,863   | 13,504   | 26,235  | 37.0        | 105               | 71                  | 30    | 4     | MT525879           |
| Passiflora watsoniana       | Subgenus Passiflorasupersection Stipulata/ section Kermesinae        | 146,520              | 86,139   | 13,355   | 23,513  | 37.0        | 105               | 71                  | 30    | 4     | MT525881           |
| Dilkea retusa               | Dilkea genus                                                         | 161,923              | 88,575   | 12,686   | 30,331  | 36.2        | 109               | 75                  | 30    | 4     | MT525866           |
| Mitostemma brevifilis       | Mitostemma genus                                                     | 163,032              | 88,837   | 12,695   | 30,750  | 36.1        | 109               | 75                  | 30    | 4     | MT525867           |

*aLoss of an IR region.*
Introns were identified in 10–15 sequences of protein-coding and tRNA genes, mainly in the Astrophea and Deidamioides subgenera (15 introns). The intron within the \textit{atpF} gene was not found in the Passifloraceae species analyzed in this study, but it was found in \textit{Po. trichocarpa}, the species used in the comparative analysis (fig. 1). Additionally, the Astrophea and Deidamioides subgenera harbored an intron in the \textit{clpP} gene, which was not found in the Decaloba and Passiflora subgenera, revealing the loss of this region. In the Decaloba subgenus, we found the lowest number of introns, because of losses in the \textit{rpoC1} and \textit{rpl16} genes.

Repetitive sequence analysis detected between 115 (\textit{Passiflora alata}) and 445 (\textit{Passiflora contracta}) repeats (table 2). Most of the repeats were found in the intergenic sequences of the LSC or IR regions, but some repeats were located in gene sequences, including \textit{accD}, \textit{clpP}, \textit{psaA}, \textit{psaB}, \textit{rps18}, \textit{ycf1}, \textit{ycf2}, and \textit{ycf3} (supplementary table S6, Supplementary Material online).

The species, the highest number of repeat structures was found in the LSC region, with some detected in the IR and SSC regions (supplementary table S6, Supplementary Material online). Comparing the subgenera, high numbers of repeats were found in the Deidamioides subgenus (a respective 405 and 445 total repeats in \textit{P. deidamioides} and \textit{P. contracta}) and Decaloba subgenus (a respective 199 and 280 total repeats in \textit{Passiflora candollei} and \textit{P. costaricensis}) in which the largest repeat (1,070 bp identified in \textit{P. costaricensis}) possibly corresponds to a piece of the IR region that was lost in this species.

Most of the repeats were found in the intergenic sequences of the LSC or IR regions, but some repeats were located in gene sequences, including \textit{accD}, \textit{clpP}, \textit{psaA}, \textit{psaB}, \textit{rps18}, \textit{ycf1}, \textit{ycf2}, and \textit{ycf3} (supplementary table S6, Supplementary Material online). Furthermore, in all species analyzed, a repeat was identified in the \textit{ndhA} gene located in the respective SSC region. Note in particular that a high number of repeats in the \textit{accD} gene and its promoter or intergenic regions was identified in all 20 cp genomes of Passifloraceae, with up to 151 repeats in \textit{P. costaricensis} (supplementary table S6, Supplementary Material online).
### Table 2
Summary of the Short Direct-Repeats Identified in the cp Genomes of 18 *Passiflora* Species and the Passifloraceae, *Dilkea retusa* and *Mitostemma brevifilis*

| Species                  | Taxonomic Classification Subgenus/ Supersections, Sections, or Series | cp Genome (bp) | REPuter Total Number | Palindromic Forward | Reverse | Complement | Largest Repeat (bp) |
|--------------------------|------------------------------------------------------------------------|----------------|----------------------|---------------------|---------|------------|---------------------|
| *Passiflora cernadensis*  | Subgenus *Astrophea*/section *Capreolata*                             | 164,515        | 118                  | 38                  | 65      | 9          | 6                  | 193                |
| *Passiflora haematostigma* | Subgenus *Astrophea*/section *Pseudoastrophea*                        | 163,775        | 399                  | 67                  | 286     | 28         | 18                 | 476                |
| *Passiflora rhannifolia*  | Subgenus *Astrophea*/section *Pseudoastrophea*                        | 162,217        | 398                  | 67                  | 222     | 56         | 53                 | 486                |
| *Passiflora candollei*    | Subgenus *Decaloba*/section *Decaloba*                                | 138,081        | 199                  | 87                  | 112     | 1          | 2                  | 201                |
| *Passiflora capsularis*   | Subgenus *Decaloba*/section *Xerogona*                               | 113,114        | 207                  | 55                  | 155     | 1          | 2                  | 791                |
| *Passiflora costaricensis*| Subgenus *Decaloba*/section *Xerogona*                               | 114,230        | 280                  | 38                  | 240     | 2          | 1                  | 1,070              |
| *Passiflora suberosa*     | Subgenus *Decaloba*/section *Ceca*                                   | 158,313        | 279                  | 108                 | 154     | 9          | 8                  | 760                |
| *Passiflora vespertilio*  | Subgenus *Decaloba*/section *Decaloba*                               | 138,456        | 238                  | 73                  | 164     | 1          | 1                  | 151                |
| *Passiflora contracta*    | Subgenus *Deidamioides*/section *Tetrastyli*                          | 166,558        | 445                  | 183                 | 262     |            |                    | 427                |
| *Passiflora deidamioides* | Subgenus *Deidamioides*/section *Deidamioides*                       | 167,953        | 405                  | 129                 | 261     | 15         |                    | 352                |
| *Passiflora alata*        | Subgenus *Passiflora*/supersection *Laurifolia*/section *Quadrangularaes* | 147,773        | 115                  | 33                  | 78      | 4          |                    | 123                |
| *Passiflora crystallina*  | Subgenus *Passiflora*/supersection *Distephana*                       | 145,054        | 128                  | 37                  | 87      | 4          |                    | 123                |
| *Passiflora edmondi*      | Subgenus *Passiflora*/supersection *Stipulata*/section *Kermesinae*    | 142,737        | 185                  | 22                  | 145     | 18         |                    | 118                |
| *Passiflora loefgrenii*   | Subgenus *Passiflora*/supersection *Stipulata*/section *Kermesinae*    | 146,537        | 133                  | 22                  | 104     | 5          | 2                  | 137                |
| *Passiflora miniata*      | Subgenus *Passiflora*/supersection *Distephana*                       | 151,920        | 152                  | 35                  | 114     | 3          |                    | 116                |
| *Passiflora mucronata*    | Subgenus *Passiflora*/supersection *Stipulata*/section *Granadiastrem* | 150,839        | 172                  | 49                  | 97      | 22         | 4                  | 372                |
| *Passiflora recurva*      | Subgenus *Passiflora*/supersection *Passifloral*/series *Passiflora*   | 151,837        | 158                  | 41                  | 116     | 1          |                    | 143                |
| *Passiflora watsoniana*   | Subgenus *Passiflora*/supersection *Stipulata*/section *Kermesinae*    | 146,520        | 158                  | 25                  | 128     | 5          |                    | 147                |
| *Dilkea retusa*           | *Dilkea* genus                                                        | 161,923        | 214                  | 91                  | 110     | 7          | 6                  | 106                |
| *Mitostemma brevifilis*   | *Mitostemma* genus                                                   | 163,032        | 398                  | 189                 | 209     |            |                    | 299                |
A Repertory of Rearrangements: Inversions, Expansions/Losses of IR Regions, and Gene Losses

The results of the genomic comparison revealed a highly rearranged structure of cpDNAs in *Passiflora*. To detect all rearrangements, *Po. trichocarpa* (Salicaceae) was used as a reference because it is a phylogenetically close species with the same cpDNA genome pattern as most angiosperms. The chloroplast genome comparison performed showed different inversions (fig. 2 and supplementary fig. S2, Supplementary Material online). In total, 22 syntenic blocks were identified among the cp genomes aligned.

Inversions were identified in the Astrophea subgenus compared with *Dilkea* and *Mitostemma*. They differed only with...
regard to one inversion (~15 kb) in the LSC region of *P. haematostigma* and *P. rhamnifolia*. In fact, a high level of synteny was found between Passifloraceae *Dilkea* and *Mitostemma*, and Salicaceae *Po. trichocarpa*, revealing that fewer rearrangements have occurred, a feature common to most angiosperms. A large inversion in the LSC region flanking the *clpP* gene was found only in *P. cerradensis*, differing from the earlier rearrangement of the *clpP* and *accD* genes in *P. haematostigma* and *P. rhamnifolia*.

Rearrangements were also found in the Decaloba subgenus, with five species exhibiting a large inversion in the LSC region (fig. 2). Small sequence structures were also found inverted in the LSC region of *P. suberosa*. Interestingly, these small inversions are different from the very distinct rearrangement located at the beginning of the LSC region of *P. candollei* and *Passiflora vespertilio*, including the *petB* and *clpP* genes. *Passiflora vespertilio* also exhibited a different arrangement in the IR region, with an inversion between the *rpl2* and *mss* genes, and this arrangement was confirmed by PCR (supplementary table S3, Supplementary Material online).

The two species of the subgenus Deidamioides (*P. deidamioides* and *P. contracta*) differed due to the presence of a small inverted block in the LSC region (fig. 2). Comparing all 20 cp genomes, the species in the subgenus *Passiflora* differed by the presence of a large inversion in the LSC region.

We also examined IR boundaries and detected different expansions and contractions (fig. 3). By comparing them with *Po. trichocarpa*, used as a reference, it was possible to detect expansions in the Passifloraceae *D. retusa* and *M. brevifilis*, in which the *ndhH* gene had expanded from the boundary of the SSC region to the IRA region (~550 bp) creating a duplicated small fragment copy of the *ndhH* gene in the IRB region. Furthermore, the LSC region contained an expansion including a portion (15 bp) of the *rpl23* sequence up to the IR region, and interestingly this arrangement seems to be particular to *D. retusa* and *M. brevifilis* and does not occur in the *Passiflora* genus, not even in the reference, *P. trichocarpa*.

In the Astrophea subgenus, as in the *Dilkea* and *Mitostemma* genera, an expansion was observed in the SSC/IRA extending into part of the *ndhD* gene, whereas *P. cerradensis* showed an additional expansion (~3.5 kb) of IRA/LSC that includes the *matK* gene, which was confirmed by PCR (supplementary table S3, Supplementary Material online). Comparing all the species sequenced, Decaloba is the subgenus with the highest number of rearrangements related to IR boundaries. For instance, in *P. candollei* and *P. vespertilio*, the expansion of IRA/LSC extends up to the *petD* gene, and in *P. suberosa* this expansion is even larger, extending up to the *psa* gene and comprising important genes such as *rbcL*. As a result of this significant expansion, *P. suberosa* has an IR of ~43 kb that includes 38 genes, a difference of 17 kb compared with the IRs of other Decaloba subgenus species analyzed in this study.

A different expansion of IRB/LSC was observed in *P. deidamioides* (subgenus Deidamioides), including the protein-coding genes *matK*, *psbA*, and *psbK*, and this expansion was confirmed by amplification by PCR (supplementary table S3, Supplementary Material online). This latter expansion in *P. deidamioides* has an additional fragment length of 3,126 bp compared with the IRB/LSC of *P. contracta* (subgenus Deidamioides). Finally, the species that belong to the subgenus *Passiflora* exhibited the smallest rearrangements related to IR boundaries, with part of the *rps15* gene expanding from the SSC to the IRA region (64 bp in *P. edmundoi*).

Comparatively, there were no large variations in the length of the SSC region, and the difference in IR sizes between species was ~21.5 kb, whereas in the LSC region it reached 33 kb. These differences were due to not only IR expansions but also gene and intron losses. All the cp genomes showed gene losses compared with the reference, *Po. trichocarpa* (fig. 1). The *rpl22* gene was not found to be complete in any of the species analyzed. Additionally, *accD* was found as a pseudogene, containing just a piece of the sequence or up to 18 stop codons in some species of the different subgenera. Some events may have occurred independently alongside chloroplast genome evolution in the *Passiflora* genus, such as *rpl32* gene loss in some species of the Decaloba and *Astrophea* subgenera (fig. 1). In addition, *rpl20* was absent in the cp genomes of the *Astrophea*, Decaloba, and *Passiflora* subgenera. In the subgenus *Passiflora*, the *rps7*, *ycf1*, and *ycf2* genes were found as pseudogenes. Finally, *Decaloba* subgenus exhibited most of the gene losses, over and above the loss of *rps7*, *ycf1*, and *ycf2* (the last two were sometimes detected as small pseudogenes), and the loss of *rpoA* was detected in *P. capsularis*, *P. costaricensis*, and *P. suberosa*.

**Phylogenomic Studies**

A phylogenomic study was performed based on 68 protein-coding genes of 49 Passifloraceae species with available chloroplast genomes. These species were used as the ingroup and *Po. trichocarpa* (Salicaceae) was the outgroup to obtain a rooted phylogenetic tree.

The two different partition schemes for Bayesian analysis had no substantial effect on the topology of the resulting trees. The analysis partitioned by codon position was highly favored in relation to the single model based on Bayes Factors (~5,448.8) and also showed slightly higher support values in nodes with PPs < 1.0. The accuracy of the inferred species phylogeny was strongly supported by the stability of the main clades generated and high PP values, with 97% (42 of 43) reaching 1 (fig. 4).
Dilkea and Mitostemma formed a clade at the position (Adenia, (Dilkea, Mitostemma, Passiflora)), all with high support in the phylogenetic tree. The Astrophea clade was supported placed on the tree as a monophyletic with high PPs = 1 and was split into two distinct subclades, one containing P. haematostigma and P. rhamnifolia (species from section Pseudastrophea) and the other grouping P. cerradensis and Passiflora pittieri. However, Passiflora arbelaezi (subgenus Deidamioides) was grouped as a sister taxon to the Astrophea species, dismembering the polyphyletic group of Deidamioides.

Deidamioides, as currently defined, is a polyphyletic subgenus, and despite the clade formed by P. contracta and P. deidamioides, other species assigned to Deidamioides were placed in different positions on the tree. Passiflora arbelaezi was placed as sister to the Astrophea subgenus whereas P. obovata (subgenus Deidamioides) was embedded in the Decaloba subgenus.
Most species from the Decaloba subgenus clustered into a monophyletic clade with high support, having as successive sister groups Tetrapathea and two species of Deidamioides (P. contracta and P. deidamioides). Subgenus Passiflora was also recovered as a monophyletic group, but its sections display paraphyletic patterns. This subgenus contains 236 morphologically diverse species, which are classified in supersections, sections, and series (Feuillet and MacDougal 2003).

**Discussion**

A Repertory of cpDNA Rearrangements Concomitant with Passiflora Evolution

The evolutionary history of the Passiflora chloroplast genome revealed a high number of structural rearrangements (fig. 5). Inversions were detected in the subgenus Astropheia (P. haematostigma), but surprisingly not in the Dilkea, Mitostemma, and even Adenia genera in the Passifloraceae

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(Fig. 4.—Bayesian phylogenetic reconstruction of Passiflora evolutionary history based on 68 chloroplast protein-coding genes.)
family (Shrestha et al. 2019). This suggests that the inversions in the Astrophea subgenus occurred after the separation of the Passiflora genus from its ancestors. The other three genera have fewer species (1–102) and exhibit cp genomes more closely resembling that of Po. trichocarpa. It is possible that the abundance of inversions occurred only in Passiflora and not in other Passifloraceae. Interestingly, compared with Po. trichocarpa, the passionflowers show two small inversions in the LSC region that could be used to characterize the species in this group.

Some inversions in the Passiflora genus could possibly be the result of intramolecular recombination of repeats, a mechanism that has been reported to impact the generation of rearrangements (Ogihara et al. 1988; Milligan et al. 1989; Gray et al. 2009; Ruhlman et al. 2017). In our study, short direct-repeat structures in the flanking regions between the accD and clpP genes were identified in both P. haematostigma and P. rhamnifolia (Astrophea). These kinds of repeats are also present in wheat (Ogihara et al. 1988), Asteraceae (Kim et al. 2005), and Geraniaceae (Guisinger et al. 2011), the latter showing highly rearranged cp genomes similar to the trends observed in Passiflora.

In addition, a distinct inversion was found in P. cerradensis (subgenus Astrophea), but because this inversion was flanked by clpP and caused repositioning of this gene, in evolutionary terms our results indicate that the rearrangement of accD and clpP genes in P. haematostigma and P. rhamnifolia occurred before the unique inversion found in P. cerradensis. Interestingly, all the inversions in Astrophea are different from those found in the other subgenera. Therefore, it is clear that Astrophea underwent further cpDNA changes after its separation from the clade ((Decaloba, Deidamioides), Passiflora) (~40 Ma) (Muschner et al. 2012).

The Decaloba subgenus exhibits many different inversions, possibly because of the high number of repeat structures, as well as large IR expansions that are typical of this subgenus. On the other hand, the Deidamioides species have a similar cpDNA structure to Dilkea and Mitostemma, with just one small inversion in the LSC region. However, the Deidamioides subgenus differs from the other two Passifloraceae in that it has large IR expansions. Finally, species from subgenus Passiflora exhibit conserved structures and the same rearrangements in the LSC region as those previously

![Fig. 5.—Evolutionary history of chloroplast genome structure in the genus Passiflora. The structure of the chloroplast genomes was plotted on a tree representing the evolution of the Passiflora genus. In the tree, each subgenus was differentiated by colors: blue for Astrophea, green for Decaloba, red for Deidamioides, orange for Passiflora, and violet for Tetrapatha. In the comparison of the cp genomes, the boxes and dotted lines indicate the direction of the structural inversions, and the red arrows indicate the direction of IR expansions/contractions. *Mitostemma brevifilis shares the same structure with Dilkea retusa, and the species of the Passiflora subgenus obtained in this study share the same structure with Passiflora alata. °cp genomes obtained from the GenBank database.](image-url)
described for *P. edulis*, the main cultivated species (Cauz-Santos et al. 2017).

Chloroplast genome IR expansions have been found in different plant groups, including Geraniaceae (Guisinger et al. 2011), Euphorbiaceae (Li et al. 2017), Solanaceae (Amiryousefi et al. 2018), and Bignoniaceae (Thode and Lohmann 2019). *Decaloba* species harbor larger variations, but *P. capsularis* lacks one of the IRs; *P. suberosa* exhibits a large expansion of the IRaLSC up to the *psal* gene. The latter IR expansions have already been described in previous studies on the *Decaloba* subgenus (Rabah et al. 2019; Shrestha et al. 2019), except for the lack of IR region in *P. capsularis* and *P. costaricensis*. In *Astrophyta* species, IRb/SSC has expanded to part of the *ndhH* gene, and remarkably an IRaLSC expansion was found to include the *matK* gene in *P. deidamioides* and *P. cerradensis*. However, these expansions do not have a common ancestor and supposedly occurred independently in the *Astrophyta* and *Deidamioides* subgenera.

All species of the *Passiflora* subgenus exhibited an expansion to the *rps19* gene, like that of *Po. trichocarpa*, but this expansion was not found in its sister group, suggesting that independent events occurred in the distinct subgenera. Expansions/contractions and loss of IRs are the main causes of variations in cpDNA sequence length, as in the *Decaloba* subgenus and other plants, such as *Annona* (Blazier, Ruhlman, et al. 2016) and *Lamprocapnos* (Park et al. 2018), as well as in monocots (Wang et al. 2008). Different mechanisms have been proposed to explain IR expansions, such as gene conversion or double-strand DNA breaks (Goulding et al. 1996; Wang et al. 2008).

Distinct genes have been lost during the evolution of cp genomes in the *Passiflora* genus, such as *rpl20* and *rpl22*, the latter absent in all the cp genomes studied herein. Because the chloroplast organelle is responsible for vital processes like photosynthesis, gene loss can impair the efficiency of some metabolic pathways, plant growth, and cell survival (Neuhaus and Emes 2000; Kode et al. 2005; Rogalski et al. 2006; Romani et al. 2012). Furthermore, *rpl20* and *rpl22* encode proteins necessary for chloroplast translational apparatus and have been proven to be essential for cell viability in tobacco knockout mutants (Rogalski et al. 2008; Fleischmann et al. 2011). Similarly, the absence of *rpl22* in legumes is offset by the existence of a functional copy transferred from the chloroplast to the nuclear genome (Gantt et al. 1991).

Gene transfer between chloroplast and nuclear genomes has been described in some species (Millen et al. 2001; Rousseau-Gueutin et al. 2013; Hong et al. 2017) and observed experimentally (Stegemann et al. 2003; Stegemann and Bock 2006; Lloyd and Timmis 2011). Despite the fact that the organelle gene needs a eukaryotic promoter to maintain its functionality in the nuclear genome, studies have revealed that some cp promoters are weakly active in the nucleus, which would render gene transfer viable without a nuclear promoter (Cornelissen and Vandewiele 1989; Wang et al. 2014). The functional transfer of *rpl22* from cp to the nuclear genome has been observed in Fabaceae and it has been suggested that this happens in *Passiflora* (Jansen et al. 2011).

The Loss of an IR Region in Subgenus *Decaloba*

The cp genomes analyzed herein have the quadripartite structure common to almost all angiosperms. However, a complete loss of one IR was identified in *P. capsularis* and *P. costaricensis*, both species in the *Xerogona* section within the *Decaloba* subgenus (Espinoza et al. 2018). It is worthy of note that this kind of arrangement has not previously been reported in *Passiflora* cpDNAs (Cauz-Santos et al. 2017; Rabah et al. 2019; Shrestha et al. 2019). Bearing in mind that the loss of a complete IR region is rare in angiosperms and it was identified in species belonging to the same section (i.e., *Xerogona*), this event may have occurred in the ancestral chloroplast genome of the *Xerogona* section (subgenus *Decaloba*).

The loss of a complete IR has been described in few species of Geraniaceae and Cactaceae (Guisinger et al. 2011; Sanderson et al. 2015; Blazier, Jansen, et al. 2016), and in the large group of Fabaceae (legumes) in which the loss of an IR occurred once, leading to a monophyletic group within the subfamily Papilionideae, designated the IR-lacking clade, or IRLC (Lavin et al. 1990). It was suggested that this IR deletion could provide a means of testing the traditional evidence used to reconstruct Papilionideae phylogeny (Lavin et al. 1990). In addition, the rates of nucleotide substitution in genes of the IRLC-papilionoids are generally higher than those found in the IR-containing papilionoids (Schwarz et al. 2017).

In land plants, IR regions were found to present slower rates of nucleotide substitution compared with those of the SC regions. However, the expansion of IRs did not necessarily decrease the substitution rates in some lineages, as in *Pelargonium* (Zhu et al. 2016; Weng et al. 2017).

Palmer and Thompson hypothesized that IR regions could play a part in the stabilization of cp genome structure (Palmer and Thompson 1982). However, later studies reported that the deletion of a complete IR would not necessarily lead to cp genome instability or the formation of new rearrangements (Palmer et al. 1987). Thus, an IR loss was considered to be a different pattern of rearrangement that occurs together with other rearrangements (e.g., inversion and gene loss) in the IRLC (Sabir et al. 2014). Interestingly, this pattern was also found in our study. The loss of a complete IR sequence in *Xerogona* section species is unique, alongside other different rearrangements that occur in the *Decaloba* subgenus, such as inversions, IR expansions, and gene and intron losses.

In *Passiflora*, previous studies revealed the occurrence of paternal inheritance in interspecific crosses (Muschner et al. 2006; Hansen et al. 2007). However, the potential for biparental inheritance in intraspecific crosses involving...
**Phylogenetic Relationships in Passiflora**

Taxonomically speaking, *Passiflora* is the largest genus of Passifloraceae with ~520 species, exhibiting high morphological diversity, especially in flower shape and color, and also in genome size. Although a wide variety of morphological traits has been applied in its traditional taxonomy, the species classification still has unresolved positions, particularly regarding species belonging to the *Deidamioides* subgenus. Molecular phylogenies did not clarify the position of the *Passiflora* (*Mraček 2006*). Further studies are required to evaluate whether changes in the inheritance of chloroplast DNA could lead rearrangements in the nucleotide sequence.

In the past, due to its morphology, *Tryphostemmatoides* was considered a subgenus (*Kilip 1938*), but later it was reduced to a section of the *Deidamioides* subgenus (*Feuillet and MacDougal 2003*). Previous studies using molecular phylogenetic inferences not only recovered section *Tryphostemmatoides* as a monophyletic clade but also revealed the paraphyletic position of this section in relation to other species of the *Deidamioides* subgenus (*Muschner et al. 2012; Krosnick et al. 2013*). Our findings also suggest the need for revisiting the taxonomic classification of *Tryphostemmatoides*.

The *Deidamioides* subgenus has been recovered as polyphyletic. Only the Brazilian endemic species *P. contracta* and *P. deidamioides* were grouped as a clade. *Passiflora arbelaezii* was sister to the *Astropea* subgenus, and *P. obovata* placed in the *Decaloba* subgenus, confirming a previously phylogenetic inference (*Shrestha et al. 2019*). The classical taxonomic studies (*Master 1871; Kilip 1938*) and the recent revision (*Feuillet and MacDougal 2003*) initially positioned some of these species in the *Deidamioides* subgenus and subsequently the *Decaloba* subgenus. Conversely, *P. deidamioides* was initially considered as *Decaloba* (*Harms 1923*), before the creation of the *Deidamioides* section. *Passiflora obovata* was considered to belong to the subgenus *Plectostemma*, now known as the *Decaloba* subgenus (*Harms 1923; Kilip 1938*), and was assigned to the *Deidamioides* subgenus in the classification of *Feuillet* and *MacDougal* (*2003*). However, molecular phylogenies still group this species in the *Decaloba* subgenus (*Krosnick et al. 2006, 2013*), corroborating our findings. This controversy suggests that the classification of *P. obovata* needs to be revised.

*Passiflora tetrandra* (subgenus *Tetrapathe*) was grouped as a sister to *Decaloba*, forming the group (*Deidamioides, Tetrapathea, Decaloba*), confirming previous findings (*Krosnick et al. 2013; Shrestha et al. 2019*). On the other hand, heteroplasmy, the existence of divergent chloroplast types, could be problematic and lead to the analysis of paralogous copies in phylogenetic studies (*Wolfe and Randle 2004*). For this reason, *Hansen et al. 2007*, discussing the implications of heteroplasmy in *Passiflora*, suggest that caution should be exercised when considering the interpretation of the chloroplast phylogenies. We should also point out that phylogenomics based on cpDNA sequences allow only one view of *Passiflora* evolution, and further reconstructions of phylogenetic trees based on nuclear genes will also be necessary.

Finally, regarding the *Passiflora* subgenus, incongruences were found in the positioning of some species compared with former phylogenetic studies. However, *Passiflora* is the largest subgenus (236 species), and due to the relevance of taxa sampling for phylogeny reconstruction accuracy (see *Heath et al. 2008; Nabhan and Sarkar 2012*), new analyses of an increased number of species may help elucidate the taxonomy.

*P. costaricensis* (subgenus *Decaloba*) has also been reported (*Hansen et al. 2007*). Most surprisingly, when faced with the cp genome structure of *P. costaricensis*, we noted that our results unveiled the loss of an entire IR region. In other groups of seed plants that exhibit potential for biparental inheritance, a highly rearranged cpDNA structure also occurs, such as in Campanulaceae (*Cosner et al. 2004; Haberle et al. 2008; Barnard-Kubow et al. 2017*) and Geraniaceae (*Metzlaff et al. 1981; Chumley et al. 2006; Weng et al. 2014*). Recent studies have shown that biparental inheritance could promote chloroplast competition mediated by accelerated rates of evolution in repeats located in the accD gene regulatory region (*Sobanski et al. 2019*). In our study, a high number of repeats within the accD gene sequence were observed in all species. Additionally, chloroplast biparental inheritance has the potential to restore cytonuclear incompatibility (*Barnard-Kubow et al. 2017; Shrestha et al. 2019*), and this type of incompatibility has also been reported in the genus *Passiflora* (*Mraček 2006*). Further studies are required to evaluate whether changes in the inheritance of chloroplast DNA could lead rearrangements in the nucleotide sequence.
within this subgenus. Interesting results were published by Sader et al. (2019) providing a robust and well-resolved time-calibrated phylogeny including some 100 taxa, and by means of phylogenetic comparative methods, they tested the relative importance of polyploidy in *Passiflora* evolution and diversification. According to these authors, changes in chromosome number and genome sizes may have contributed to morphological and ecological traits that explain the pattern of diversification observed in the genus.

### Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

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