Plukenetia volubilis non-paternal oDNA transmission: a cytological diagnosis of pollen grains and a phylogenetic approach assessing Colombian cultivars cpDNA markers variation

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Abstract

Plukenetia volubilis (Malpighiales: Euphorbiaceae) also known as Sacha inchi is a promissory crop given its high seed content of unsaturated fatty acids (UFAs) and collection of essential amino acids. The last decades, *P. volubilis* has been establishing in Antioquia, Colombia, suggesting possible adaptations to this rather new environment. However, little is known about its genetics. Here, we aimed (i) to unveil the chloroplast DNA (cpDNA) inheritance mode of *P. volubilis* by 4′,6-diamidino-2-phenylindole (DAPI) staining directly over the pollen grains after pollinic tube germination, (ii) to trace the cytoplasmic DNA fate during microsporogenesis analysing histological slices of different male flower buds developmental stages. Since organellar DNA (oDNA) could only be ultimately verified by tracking traits in large progeny samples, we also aimed (iii) to phylogenetically analyze the variability of cpDNA trnH^{GUG}-psbA intergenic spacer (IS) sequences of five *P. volubilis* cultivars from Antioquia, Colombia, in order to determine the ideal parents for upcoming crossbreeding studies. Staining results of germinated pollen grains displayed no oDNA during male germination unit (MGU) migration through pollinic tube. Furthermore, cytological diagnosis of histological slices showed cytoplasmic DNA decay from flower buds developmental stage III to IV, that is during first pollen mitosis (PMI), and between mature pollen and its germination. These observations strongly suggest that (i) *P. volubilis* cpDNA and mitochondrial DNA (mtDNA) are not paternally transmitted and (ii) that male oDNA loss might be due to a gamete exclusion mechanism during PMI coupled with perhaps a digestion system. In addition, phylogenetic analysis of the trnH^{GUG}-psbA among South American *P. volubilis* cultivars verified the divergence between cultivars from different countries showing high supported bayesian posterior probability values. Thus, (iii) the trnH^{GUG}-psbA IS from *P. volubilis* cpDNA has intraspecific resolution, but not enough to contrast local cultivars. Sequences alignment might be suitable to perform molecular analyses in progenies towards ultimate proof of cpDNA inheritance mode of *P. volubilis*.

Key words

Plukenetia volubilis, Sacha inchi, oilseed crop, oDNA, cpDNA, chloroplast inheritance, DAPI, intraspecific cpDNA marker.

Introduction

Plukenetia volubilis L. is a climbing, perennial, semiwoody, twining oilseed plant of the Euphorbiaceae family, endemic to tropical Peruvian Amazonia that grows mainly in tropical forests at altitudes between 200 and 1500 AMSL (Gillespie, 1993; Krivankova et al., 2012). Morphologically, Gillespie (1993) remarks that the principal synapomorphies of *Plukenetia* genus are the four-carpellate ovary and the associated character of four pistillate sepals. Likewise, *P. volubilis* is distinguished from the others neotropical species (11 *spp.*) by styles

mostly fused into cylindrical column (Gillespie, 1993). Besides, *P. volubilis* is becoming recognized worldwide given its outstanding nutritional profile (Hamaker et al., 1992; Chirinos et al., 2013). Some efforts have been made towards assessing *P. volubilis* crop potential capacity to become an important dietary source (Chirinos et al., 2013). For instance, its amino-acid profile presents higher fractions of tryptophan, cysteine, tyrosine than others oil-seeds sources, reaching FAO highest standards for 2-5 years children dietary supplements, except for leucine and lysine (Hamaker et al., 1992). Importantly, it has been shown that the crop relevance of *P. volubilis* truly rely on its high seed unsaturated fatty acids (UFA) content, particularly of α-linolenic acid or ω3 (12.8 - 16.0 g/ 100 g seed) and linoleic acid or ω6 (12.4 - 14.1 g/100 g seed) (Chirinos et al., 2013).

Studies done in plants suggest that *de novo* UFA synthesis pathway dependent of acyl carrier protein (one of the two main pathways involved in storage lipid accumulation) occurs virtually only in plastids (Wang et al., 2012). Indeed, chloroplasts genome harbor essential UFA synthesis pathway genes (e.g. FAD6 encoding for the oleate desaturase) and perform an important role as compartment for the FAs biosynthesis (Wang et al., 2012). However, *P. volubilis* chloroplasts genetics have not been deeply studied. Particularly, inheritance mode of its oDNA remain unknown. In all plants taxa, there are three possible ways by which oDNA could be inherited: maternally, paternally and biparentally. Angiosperms display mainly a maternal inheritance mode of its chloroplasts (Corriveau and Coleman, 1988; Greiner et al., 2015). However, new studies have shown that some of them are indeed showing a potentially biparental plastid inheritance (PBPI) mode (i.e. the tendency of inherit organelle genomes from both parents) (Zhang. 2010). Since cpDNA paternal contribution in angiosperm hasn't been reported extensively and mitochondrias are quite imprescindibles during pollen tube germination (Twell et al., 2006), we believe that *P. volubilis* cpDNA is maternally inherited and mtDNA is biparentally inherited. Which would appear suitable to develop genetically modified variants harboring cpDNA modifications avoiding gene scape and, as it has been shown in cpDNA enhanced plants displaying higher expression of proteins allowing them to overcome biotic and abiotic stress (Glick et al., 2010, *P. volublis* might be a good alternative towards this direction.

Here we approached to the inheritance mode of *P. volubilis* oDNA cytologically: using the DAPI stain method on germinated pollen grains and on histologically cut flower buds to i) determine whether or not oDNA is present in final stages of *P. volubilis* male gametes and ii) elucidate the mechanisms by which the inheritance mode of *P. volubilis* oDNA is carried out. Additionally, we used a phylogenetic approach to test the trnH^{GUG}-psbA IS, a promissory cpDNA marker based on its PIC (Shaw et al., 2007), in a set of five geographically distinct Colombian cultivars along with other South American *P. volubilis* cultivars to define possible crossbreeding parents for further studies.

Materials and Methods

Sample collection

Five cultivars from Antioquia; Colombia showing different ecological habitats such as grasslands, savannas, shrublands, conifer forest and rainforest, and ranging in an altitudinal gradient from 685 to 1501 AMSL were selected (Table 1). Both, leaves and seeds were

collected from individuals at each cultivar, washed completely with distilled water, wiped and packed in bags to avoid light degradation, and stored at -20 °C. Additionally, flower buds from Santa Rosa Cultivar (C1) were sampled in their first four developmental stages according to Cachique (2006) to cytological observations.

Pollen preparation and DAPI staining

Most developed flower buds (i.e. stage IV, just before anthesis. See (Cachique, 2006)) from different individuals were immersed in a beaker with 5 % sucrose overnight, later dried with towel paper. Then, water excess was absorbed by laying out the flower buds into silica gel inside a desiccation chamber. Later, a Zeiss Stemi DV4 stereo microscope were used to observe and taping dehiscent flower buds anthers over well depression slides to release the pollen. Afterwards, two drops of germination media (GM) [Sucrose 10%, boric acid (100 mg/L), Magnesium sulfate (200 mg/L), Potassium nitrate (100 mg/L)] (modified from Brewbaker and Kwack, 1963) were spilled into the wells. Well depression slides were stored in a dark chamber at 25 ± 3 °C for 24 h. Slide preparation for DAPI (Life Technologies, R37606) staining was carried out following three approaches: (i) pollen grains samples were left dried at room temperature before staining in the GM. (ii) ixation process in which pollen grains samples were treated sequentially with 10 % and 30 % ethanol, and then incubated at 36 °C to speed up evaporation. (iii) slide preparation flower buds in four developmental stages were dehydrated by rinsing with glutaraldehyde and successive 20 %, 40 %, 60 %, 70 % and 96 % ethanol solutions (20 min each), then dried in a BD23 Binder incubator at 37 °C. Subsequently, histological sections of flower buds were carried out embedding them in paraffin (Paraplast, 39601006, Leica) and cut at 10 µm with a microtome (RM2125 RTS, Leica). Flower buds histological slides were dehydrated at 65 ºC in a BD 23 Binder incubator for 1 h and then immersed in a succession of ethanol concentrations (60 %, 40 % and 20 %, 3 min each) for rehydration. Finally, slides were dehydrated with a succession of alcohols (20 %, 40 % and 60 %, 3 min each) and rinsed with one drop of DAPI solution and then dried for 1 h. Images of pollen grains and histological sections were first processed using ZEN software from AxioCam Carl Zeiss and finally with FIJI (2012).

DNA extraction and PCR conditions

Total DNA from leaf tissues of cultivars (C1-C5) was extracted using CTAB 2X method (Doyle and Doyle, 1982) and adapted from Villanueva-Mejía et al. (2015). Integrity and concentration of DNA extractions were evaluated in the Nanodrop 2000 (Thermo Scientific). TrnH^{GUG}-psbA IS of each cultivar cpDNA was amplified in a Bio-Rad thermal cycler C1000 TouchTM with the following primers: trnH^{GUG} (5’-CGCGCATGGTGGATTCACAATCC-3’) (Tate and Simpson, 2003) and psbA (3’-GTTATGCATGAACGTAATGCTC-5’) (Sang et al., 1997). PCR reagents concentrations for 25 µL of reaction were: Buffer taq 1X, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, trnH^{GUG} 0.5 µM, psbA 0.5 µM, DNA 100 ng, Taq polymerase 1 U adapted from Cardinal-McTeague y Gillespie (2016). The following PCR profile was used: initial denaturalizing at 94°C for 3 min, then 34 cycles of (i) denaturalizing at 94 °C for 45 s (ii) annealing at 64.5 °C for 60 s and (iii) extension at 72 °C for 2 min, finally an extension at 72 ºC for 5 min (Cardinal-McTeague and Gillespie, 2016). PCR products were checked in a 1 % agarose gel and stored at -20 ºC.

Sequencing and phylogenetic analysis

Amplified products from trnH^{GUG}-psbA IS (~ 500 bp) were sequenced using Sanger method (Macrogen inc., Korea). Eventually, sequences were trimmed for base scored below 18 phred score in a 5 base window average using Biopython libraries (Cock, 2009) (see supplementary material). Then, each trnH^{GUG} was locally aligned with the reverse complement of its psbA cultivar sequence. Each cultivar consensus region was reconstructed using the Smith-Waterman algorithm (1981), with an identity greater than 95 %. Thenceforth, the identity of the species fragments were verified running BLAST algorithm in the NCBI database (see supplementary material) and sequences were deposited in the genbank with the accession numbers pending. Subsequently, these sequences and all *P. volubilis* and *P. lehmanianna* available for this región were retrieved from NCBI and were aligned using ClustalW on Geneious Pro ver. 11.1.5 (http://www.geneious.com) and analyzed the aligned matrix with JmodelTest software ver. 2.1.10 (Darriba et al., 2012). TPM1uf (Kimura, 1981) substitution model was selected based on the delta Akaike Information Criterion (ΔAICc) and delta of Bayesian Information Criterion (ΔBIC) information criteria. BEAST ver. 2.5 were used to reconstruct the phylogeny of our nucleotide matrix. The Markov Chain Monte Carlo (MCMC) algorithm was run for 10,000,000 generations, sampling one tree every 1000 generations. Finally, the best topology was visualized using FigTree ver. 1.4.3.

Results and discussion

Cytological analyses

To elucidate the oDNA inheritance mode of *P. volubilis* a cytological approach using DAPI staining was implemented in three steps: (i) direct DAPI satin into germinated pollen grains; (ii) DAPI stain on alcohol fixed germinated pollen grains and (iii) DAPI staining on histological slices of four flower buds developmental stages. During pollen grain germination (approaches i and ii), appearance of three aperture furrows is conspicuous, these furrow expose feeble intine to the GM and are the proper places for tube germination (Figure 1A). Besides, observations of germinated pollen grains of *P. volubilis* with DAPI stain under fluorescent microscopy showed no evidence of plastid DNA or any oDNA granules in any of the germination stages observed (Figure 1), therefore this study appears to be the first cue suggesting that *P. volubilis* oDNA is not paternally transmitted.

Given that cytological diagnosis of oDNA is conducted in male gametic cell, that is, in a prezygotic state, then, it's still unknown oDNA fate during fertilization. For instance, in *Ephedra distachya* very few sperm nuclei are associated with the plastids and mitochondria and they are degenerating during fertilization (Zhong et al. 2011), showing that presence of plastid in ultimate states of male gametic cell could be interpreted as false positives. However, to date it has been shown, based on different approaches but mainly cytologically (scanning plastid DNA in the male gametic cell with DAPI), that 80 % of angiosperms display maternal inheritance mode of cpDNA, while the remaining 20 % of angiosperms studied show a strong tendency for plastid transmission from the male lineage, a phenomenon known as potential biparental plastid inheritance (PBPI) (Corriveau and Coleman, 1988; Zhang, 2010). On the other hand, paternal inheritance in angiosperms has

been only observed in barely some cases (Harris and Ingram, 1991; Testolin and Cipriani, 1997).

Table 1. Cultivars sampling points. Altitudes range from 685 to 1501 AMSL and habitat vary among all cultivars. Both, leaf tissue and seeds were collected.

Cultivar	Town	Altitude (AMSL)	Habitat	Coordinates
C1	Santa Rosa	1501	Grasslands, savannas, shrublands	6° 35' 42,9" N, 75° 13' 49,6" W
C2	Caucasia	44	Grasslands, savannas, shrublands	7° 58' 58,9" N, 75° 10' 4,1" W
C3	Yarumal	1118	Rainforest	7° 2' 31,3" N, 75° 21' 5,6" W
C4	San Francisco	561	Conifer forest and rainforest	5° 55' 14,2" N, 74° 52' 5,8" W
C5	San Luis	685	Conifer forest and rainforest	6° 1' 30,7" N, 74° 58' 6,1" W

Hence, plant cytoligsts agree with considering DAPI staining method as if not ultimate evidence, a very accurate approach, arguing that migration of MGU through pollinic tube could be considered the final and active state of gametophyte (Twell et al., 2006). Then, if no plastids are present during the migration of MGU, it is reasonable to think there will not be paternal plastids in the zygote (Zhong et al., 2011). However, constraints in terms of sampled number to determine inheritance mode are important because plastid inheritance is a continuous phenomenon and it is well known that degree of certainty of maternally inherited plastids can only be increased reaching the peaks in a binomial distribution of crosses (Milligan, 1992). In other words, oDNA could be paternally transmitted in non-sampled individuals within the species, bringing up the possibility of paternal inheritance in *P.volubilis* as in other species (Corriveau and Coleman, 1988).

Furthermore, diagnosis of pollen grains fixed with alcohol (Figure 1. C-D) finely displaying the movement of the MGU is the first study known to date showing that *P. volubilis* has a binucleate male gametic cell. It exhibits a diffuse nucleus (vegetative) and a more defined one (germinative cell) at the end of gametogenesis. Despite almost every plant species show binucleated stereotypical pollen grains (70 % of all plant *spp*. according to Twell et al. (2006)), trinucleate or binucleate pollen is a random feature among Euphorbiaceae (Brewbaker, 1967), therefore rendering a useful characteristic regarding pollen based taxonomy.

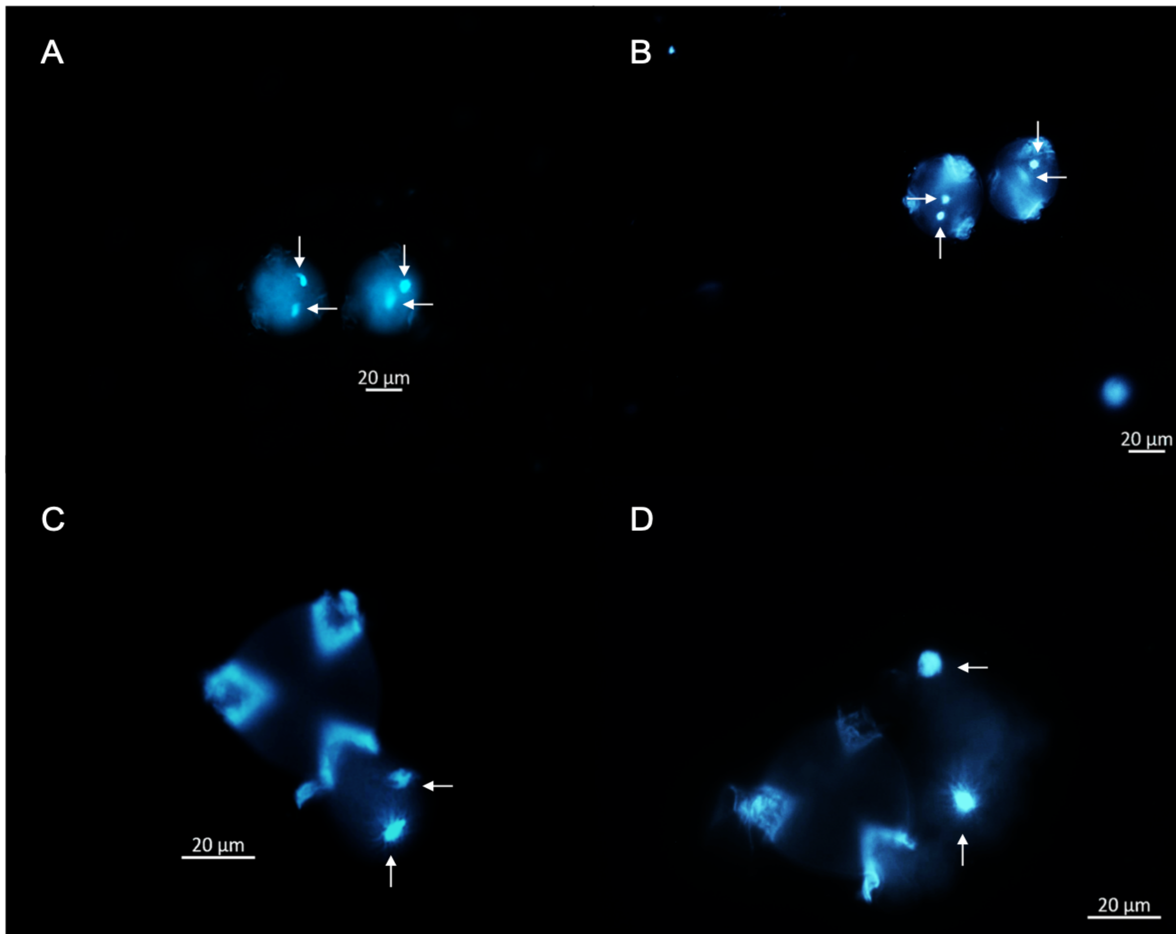

Figure 1| Germination stages of *P. volubilis* pollen grains using GM, stained using DAPI fluorochrome and visualized under fluorescent microscopy. **A** (stage 1), pollen grains not complete germinated, lacking pollen tube and stained directly. **B**. (stage 2), pollen grains showing very conspicuous germination aperturas. **C** (stage 3) pollen partially germinated and observable migration of vegetative and germinative cells. **D** (Stage 4), pollen grains germinated and conspicuous pollen tubes with male germination unit (MGU) migrating across the tube. Germinative cells are illustrated by vertical arrows and vegetative nuclei are pointed by the horizontal arrows. **A** and **B** were observed at 20X. **C** and **D** were observed at 40X.

On the other hand, when considering plastid DNA or oDNA granules, mtDNA could be included as well. Therefore, since DAPI staining is a non-selective DNA stained method and no stained plastids were observed (Figure 1), then *P. volubilis* mtDNA inheritance mode seems to be non-paternally as well. Constraints on this remark are analogous to cpDNA inheritance mode ones; that is mtDNA might be hidden in nuclei and there is a small sample of individuals. Despite constraints of mtDNA transmission are similar, mtDNA is independently trafficked from that of cpDNA. For instance, cpDNA and mtDNA of *Medicago sativa* (alfalfa) are inherited biparentally and maternally, respectively (Forsthoefel et al. 1992), whereas those of *Musa acuminata* (banana) are inherited maternally and paternally, respectively (Fauré et al. 1994). These oDNA behaviors correlate well with the degradation or amplification of DNA in each organelle of the generative cell (Nagata et al. 1999); in the generative cells of *M. sativa*, cpDNA is amplified while mtDNA

is degraded, whereas in *M. acuminata*, cpDNA is degraded while mtDNA is amplified (Twell et al., 2006).

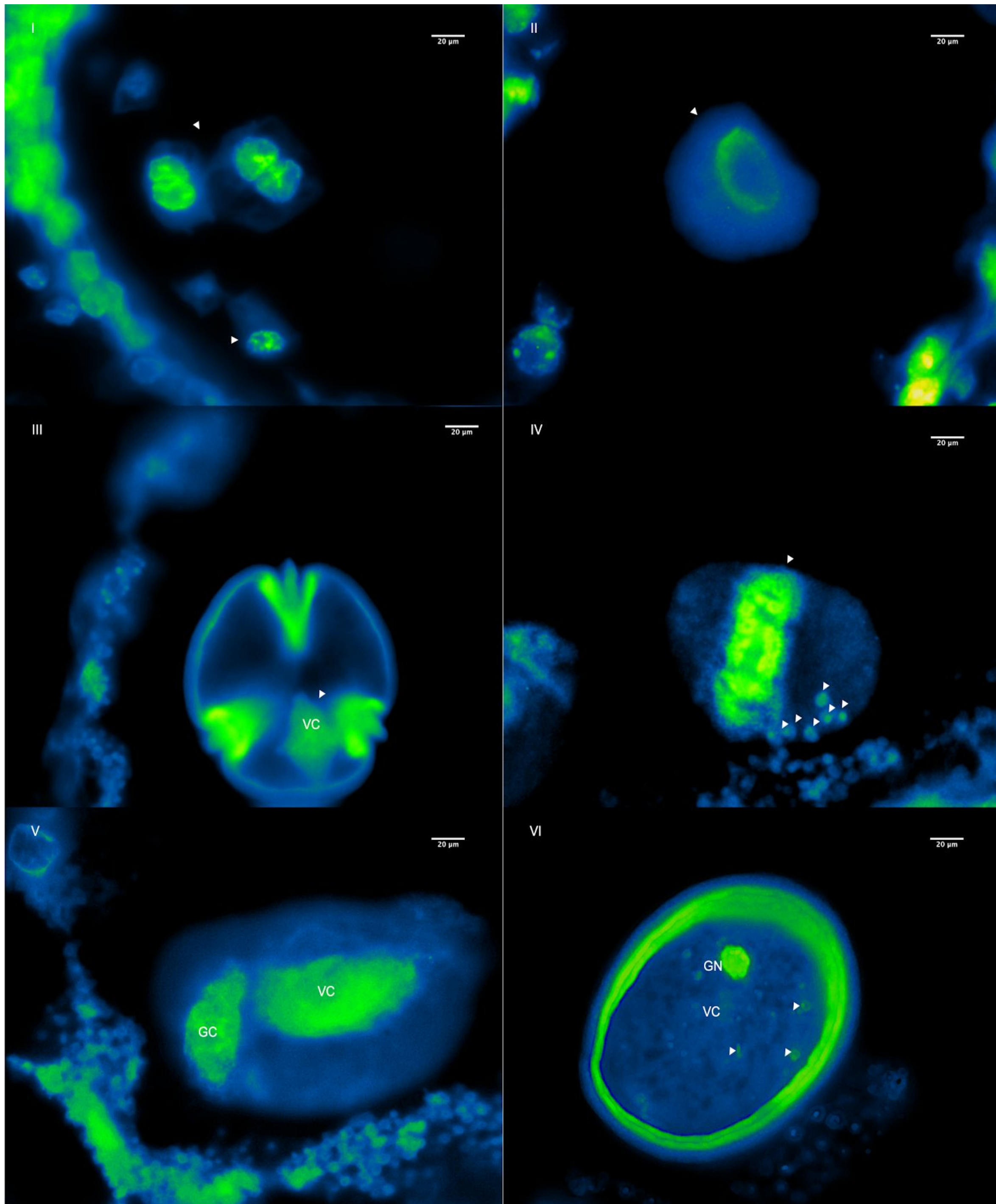

Figure 2| Six morphological stages of microsporogenesis and microgametogenesis are shown during the first four flower buds developmental stages of pollen cells of *P. volubilis* using fluorescent micrograph and DAPI stain. : **I** First meiosis and formation of tetrads, **II** Microspore release, **III** Microspore during interphase, **IV** Microspore undergoing the first pollen mitosis (PMI) and trafficked fluorescent oDNA granules (pointed out with the arrows), **V** Formation of generative cell (GC) and vegetative cell (VC), **VI** Mature pollen

and cytoplasmic DNA decay and formation of the final MGU before anthesis. All observations were carried out at 100X.

First results obtained suggest that oDNA in *P. volubilis* is not paternally transmitted. However, no information about mechanism could be deduced from this conclusion. To analyze the course of oDNA along pollen gametogenesis, histological slices of flower buds during four developmental stages were stained with DAPI. Results showed oDNA decay during switch of flower buds developmental stages III to IV (Figure 2). This decrease might be happening during PMI. Loss mechanism are several, from restriction enzyme atack to total suppression of cytoplasmic content in male gametophytic cell (Sager and Lane, 1972). Indeed, many cellular mechanisms controlling oDNA inheritance transmission have been proposed clearly, for instance: (1) physical exclusion of the organelle itself during PMI; (2) elimination of the organelle by formation of enucleated cytoplasmic bodies (ECB); (3) autophagic degradation of organelles during male gametophyte development; (4) digestion of the organelle after fertilization; and (5) digestion of oDNA in generative cells just after PMI (Nagata, 2010). However, hypotheses (1) and (5) may encompass others and also explain better the maternally or non-paternally transmitted mechanism of oDNA (Nagata, 2010).

Since results showed an organelle decay in the very early stages of mitosis (i.e. PMI) (Figure 2 IV-VI) there is a strong chance that oDNA loss mechanism in *P. volubilis* is primarily due to a organelle physical exclusion system. Indeed, many granular bodies are seen bordering outside pollen grains (Figure 2 IV), suggesting an oDNA removal from pollen grains. Nonetheless, this observations cannot conclude this hypothesis till molecular studies show how actin filaments or microtubule traffick organelle during microgametogenesis (Twell et al., 2006). Furthermore, loss mechanism may not be mutually exclusive, so multiple systems might be acting during pollen formation. In fact, it is reasonable to think that later, between the last stage of pollen formation (Figure 2 VI) and pollen germination (Figure 1 C-D), a second mechanism such as digestion of organelle may be happening. Finally, observations of histological slides should be carefully taken while they could eclipse real phenomena because tridimensional bisected pollen grains may exhibit a picture imbalance of what it is inside, just as a halved sphere piñata may result in a full candy half and an empty one.

Phylogenetic analysis

TrnH^{GUG}-psbA IS was primary selected based on its number of nucleotide substitutions or potentially informative character (PIC) value relative to other regions of the cpDNA because it presumably shows considerable variability (> 50 %) between angiosperm lineages (Shaw et al., 2007). To evaluate the resolution of the cpDNA trnH^{GUG}-psbA IS and untangle intraspecific variation in *P. volubilis* among Colombian cultivars, total DNA was extracted from leaf tissues of five plant cultivars across Antioquia, Colombia displaying ecological and altitudinal variation (Table 1). Next, the cpDNA marker trnH^{GUG}-psbA IS was amplified and sequenced through Sanger technology.

Table 2 Cultivars sequences window between nucleotides 321 and 345 displaying variations. San Luis cultivar (C5) shows important indel of 6bp and three possible single nucleotide variants (SNV). Dots (.) represent homologous bases while dashes (-) represent deletions.

Cultivar	Sequence (trnH^{GUG}-psbA)
	321 **345**
Santa Rosa (C1)	· · · · · · · · · · · - - - - - - · · · · · · · ·
Caucasia (C2)	· · · · · · · · · · · - - - - - - · · · · · · · ·
Yarumal (C3)	· · · · · · · · · · · **T** - - - - - - · · · · · · ·
San Francisco (C4)	· · · · · · · · · · · - - - - - - · · · · · · · ·
San Luis (C5)	· · · **G** · · · · · · · **T A T T T A** · · · · · · **T A**

Alignments of trnH^{GUG}-psbA IS sequences revealed nucleotide variation (Table 2). Particularly, San Luis cultivar (C5) sequence showed múltiple insertions between the nucleotides 321 and 345, while C1-C4 had a deletion in the same region (Table 2). This result not only confirm the variability of the cpDNA trnH^{GUG}-psbA IS intraspecifically, but also demonstrates the suitability of the cpDNA marker to distinguish among the local *P. volubilis* cultivars by the use of molecular techniques such as PCR coupled to Restriction Fragment Length Polymorphism (RFLP) given its polymorphisms. Many agents could be explaining the exhibited variability of *P. volubilis.* For instance, it has been shown that the diverse ecological structure of the Peruvian Amazonia has allowed domestication of native plants (such as *P. volubilis*) keeping a high genetic variability during the last centuries (Rodríguez et al., 2010). In this context, unprecedented dispersion of *P. volubilis* as a promissory crop through different regions of South America might be possible generating new varieties adapted to the different habitat conditions (Correa and Bernal, 1990; Gillespie, 1993). Besides, spotted SNVs (table 2) can be tested for simultaneous occurrence in an *P. volubilis* interbreeding population and serve as additional statistical data supporting single nucleotide polymorphism (SNP) discovery.

To evaluate the cultivar sequences from a bioinformatic perspective, we performed a phylogenetic analysis with an aligned matrix (all Colombian cultivars + four South American accessions + *P. lehmanniana* as an outgroup) (Figure 3). Surprisingly, according to the phylogenetic hypothesis obtained, no differences among Colombian cultivars is observed (Figure 3). Notwithstanding, other relationships between Peruvian and Bolivian, and Equatorial accessions of *P. volubilis* (Figure 3) seem to display important divergence. Furthermore, *P. lehmanniana* as sister group are consistently with previous studies (Cardinal-McTeague and Gillespie, 2016). Remarkably, the three main groups diverged

with posterior probability values greater than 0.98 (Figure 3), which illustrates trnH^{GUG}-psbA proper resolution for intraspecific relationships, but with some restraints whenever analysing very closely related cultivars.

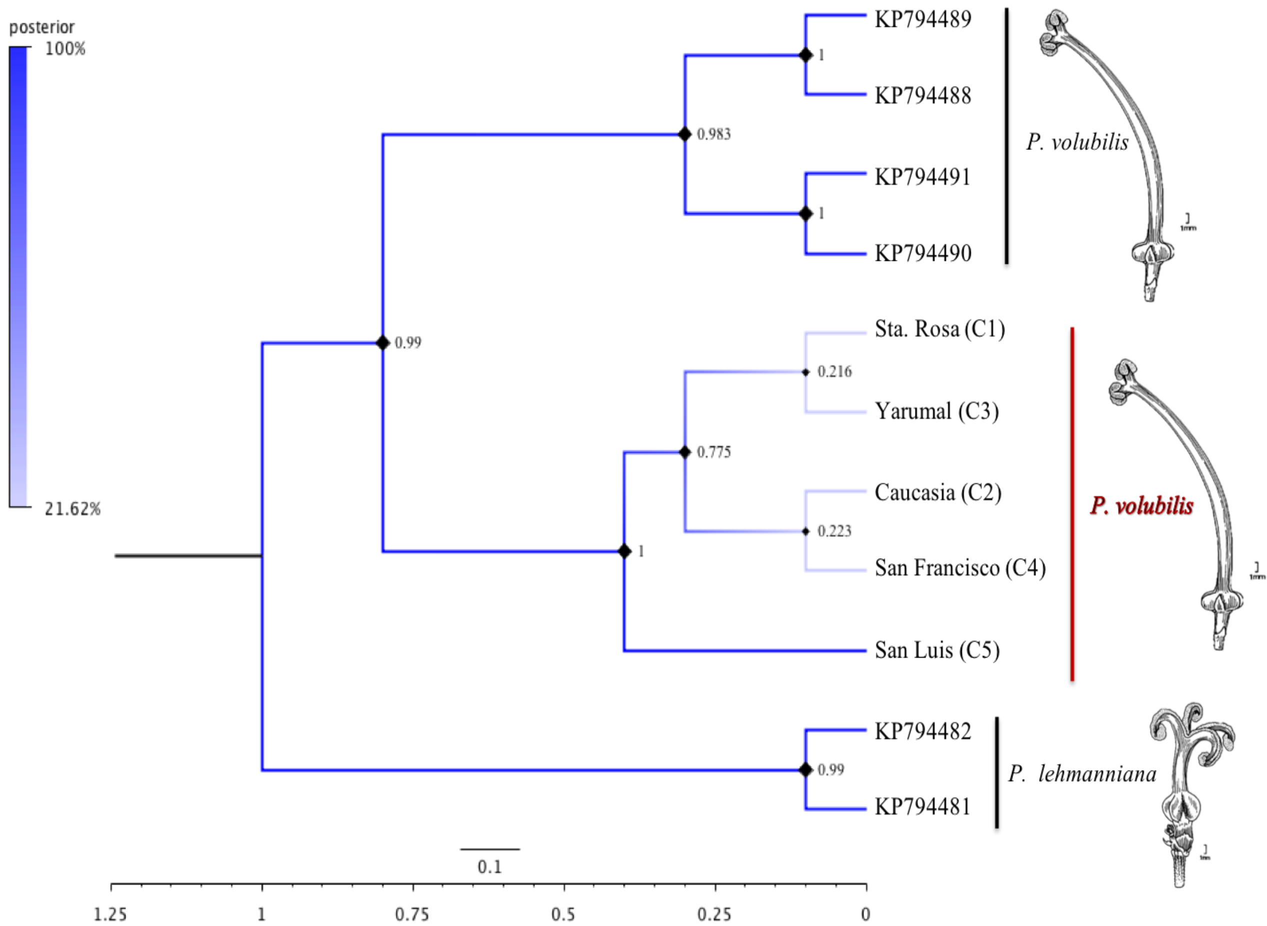

Figure 3| Phylogenetic hypothesis based on trnH^{GUG}-psbA IS of the *P. volubilis* cultivars from Antioquia, Colombia, its relatives (KP794489-91 from Bolivia, Perú and Ecuador, respectively. See (Cardinal-McTeague and Gillespie, 2016)) and selected outgroups (KP794481-82 representing *P. lehmanniana*), the MCMC algorithm was run for 10,000,000 generations, sampling one tree every 1000 generations using the TPM1uf (Kimura, 1981) evolutionary substitution model. Node support is represented with posterior probability and lower bar represents the nucleotide substitutions. Branches with high support are darker and poor supported ones are shaded. Illustrations from Cathy Pasquale were adapted from Gillespie (1993).

Conclusions

Cytological observations on germinated pollen grains suggest for the first time that *P. volubilis* oDNA is not paternally transmitted. However, in order to give additional support to these observations, analyses of oDNA transmission must be done on large progenies to obtain statistical certainty of maternal transmission mode. Additionally, this study is the

primary report showing that *P. volubilis* has a binucleated gametophyte, which may be an important taxonomic trait.

Histological observations of *P.volubilis* flower buds showed that oDNA mostly disappear after PMI, which is theoretically consistent with a physical exclusion of organelles. However, others mechanisms might be happening such as digestion of oDNA in turn and also may be acting independently for mtDNA and cpDNA. More studies must be directed to understand this phenomena from gene expression to to cell biology approaches.

The trnH^{GUG}-psbA IS from cpDNA is a suitable marker to reveal intraspecific relationships among *P. volubilis* cultivars, but not very resolutive to reveal very closely related cultivar relationships (i.e from the same región). In this study we found no differences among local cultivars. Despite this result, alignments from local cultivars may be suitable to perform molecular restrictions analyses, such as PCR-RFLP and allow us to postulate it as distinguishable parent for crossbreeding studies.

Abbreviations

ΔAICc: Akaike Information Criterion; Δ**BIC**: delta of Bayesian Information Criterion; **AMSL**: Above Mean Sea Level; **cpDNA**: Chloroplast DNA; **DAPI**: 4′,6-diamidino-2-phenylindole; **GC**: Generative Cell; **GM**: Germination Media; **IS**: Intergenic Sequence; **MCMC**: Markov Chain Monte Carlo; **MGU**: Male Germination Unit; **mtDNA**: Mitochondrial DNA; **oDNA**: Organellar DNA; **PIC**: Potentially Informative Character; **PBPI**: Potential Biparental Plastid Inheritance; **SNV**: Single Nucleotide Variant; **SNP**: Single Nucleotide Polymorphism; **UFA**: Unsaturated Fatty Acids; **VC**: Vegetative Cell.

Authors contribution

CG and FG contribute equally on this work by collecting processing and analysing pollen samples and then observing, capturing and processing images. CG and FG and VR contributed performing the DNA extractions, PCR experiments. CG and FG performed the bioinformatic analyses. On the writing CG and FG totally contributed. JC and VR guided every step of the experiments and helped with invaluable and thoroughly suggestions.

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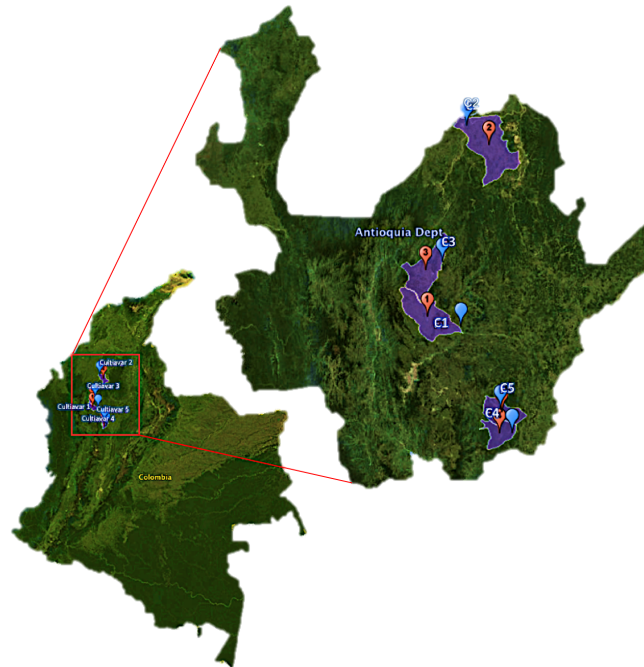
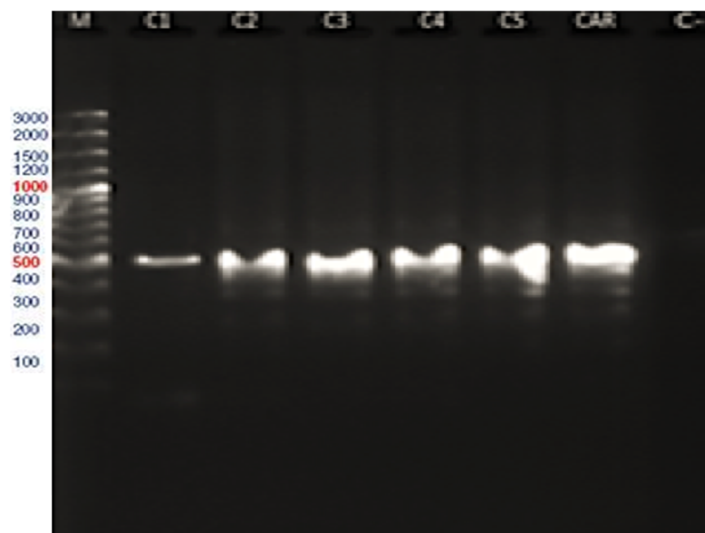
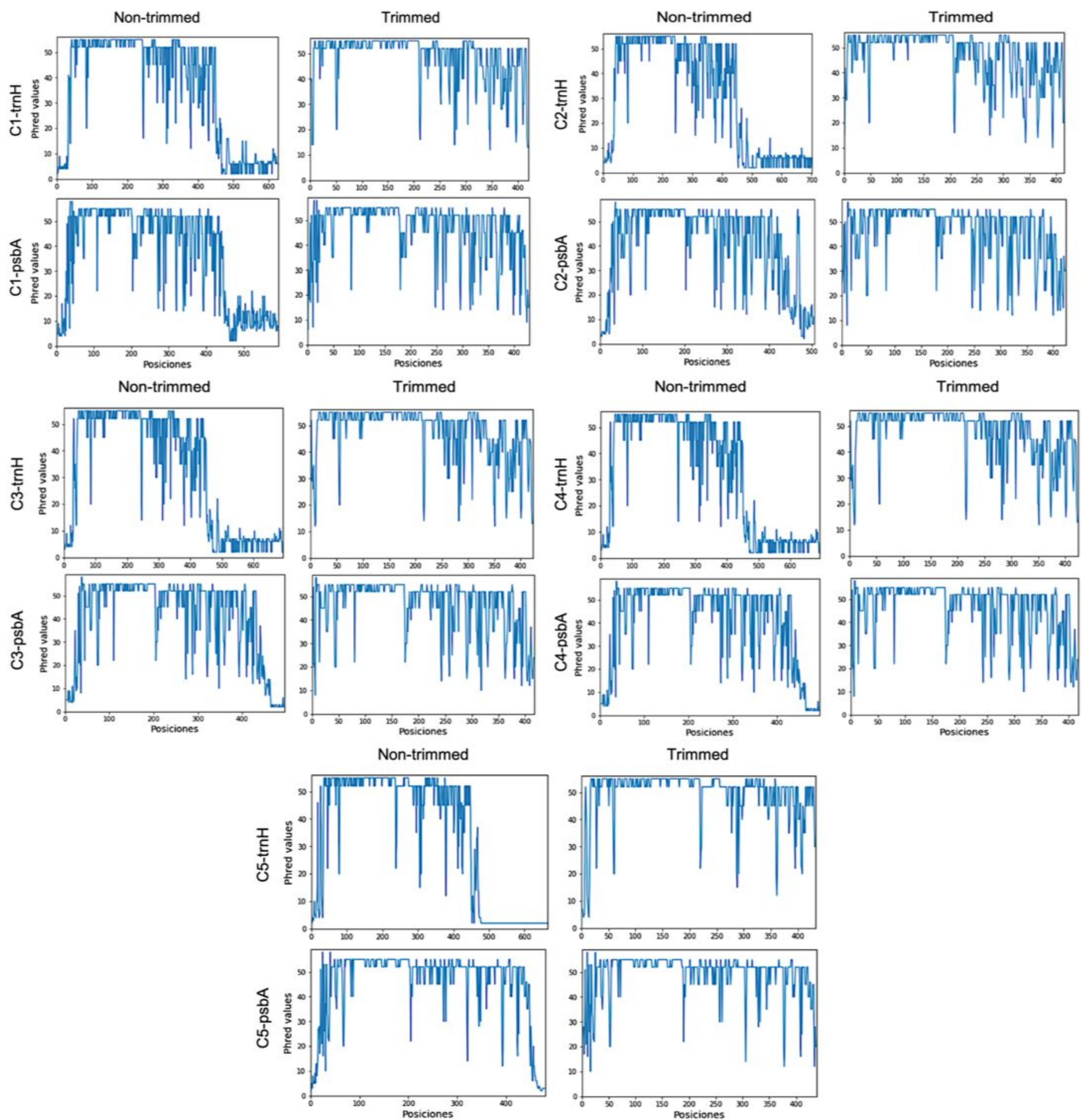
Supplementary material



Supplementary Figure S1| Geographical sample points of cultivars farmlands across Antioquia, Colombia. Labels 1-5 correspond to the farmland town closer to the collection site of the cultivars (C1-C5). C1: Santa Rosa de osos, 6° 35' 42,9" N, 75° 13' 49,6" W; C2: Caucasia, 7° 58' 58,9" N, 75° 10' 4,1" W; C3: Yarumal, 7° 2' 31,3" N, 75° 21' 5,6" W ; C4: San Francisco, 5° 55' 14,2" N, 74° 52' 5,8" W; C5: San Luis, 6° 1' 30,7" N, 74° 58' 6,1" W. Google Earth ©.



Supplementary Figure S2| Amplifications of the trnH^{GUG}-psbA IS of the five cultivars following the steps mentioned in the methodology.



Supplementary Figure S3| Sequences nucleotides vs. phred score. Trimmed sequences were selected for phylogenetic studies after treatment using Biopython library to trimme them for nucleotides under 18 phred score in a 5 nucleotides window.

Supplementary Table 1| Identity of sequences in Genbank by recalling each sequence after trimming.

Cultivar	Access number	Name	Identity (%)
C1 trnH	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	97.746
C2 trnH	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.033
C3 trnH	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	97.796
C4 trnH	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	97.857
C5 trnH	KP794489.1	*Plukenetia volubilis* isolate WCM_61 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.360
C1 psbA	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.356
C2 psbA	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.082
C3 psbA	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.082
C4 psbA	KP794491.1	*Plukenetia volubilis* isolate GIL_769 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.356
C5 psbA	KP794489.1	*Plukenetia* volubilis isolate WCM_61 photosystem II protein D1 (psbA) gene, tial sequence; psbA-trnH intergenic spacer, complete sequence; and tRNA-His (trnH-GUG) gene, partial sequence; chloroplast	98.921