



## Research article

# A quantitative PCR approach for determining the ribosomal DNA copy number in the genome of *Agave tequilana* Weber



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## ABSTRACT

**Background:** *Agave tequilana* has a great economic importance in Mexico in order to produce alcoholic beverages and bioenergy. However, in this species the structure and organization of the rDNAs in the genome are limited, and it represents an obstacle both in their genetic research and improvement as well. rDNA copy number variations per eukaryotic genome have been considered as a source of genetic rearrangements. In this study, the copy number of 18S and 5S rDNAs in the *A. tequilana* genome was estimated, and an absolute quantitative qPCR assay and genome size was used. In addition, an association between the rDNAs copy number and physical mapping was performed to confirm our results.

**Results:** The analysis were successfully applied to determine copy number of 18S and 5S rDNAs in *A. tequilana* genome, showing high reproducibility with coefficient of variation (CV) values of 0.014–0.0129%, respectively. A variation of 51 times in the copy number the 18s regarding 5s rDNA was found, thus contributing to genome size of 1.47 and  $8.38 \times 10^{-3}\%$ , respectively. Similarly, data show a linear relationship ( $R [2] = 0.992$ ) between rDNA copy number and the detected signals for each of the loci by FISH. The comparison of the rDNA copy number of agave showed differential relationship with other organisms and it may be due to evolutionary ecology.

**Conclusions:** Results show that the proposed method a) can correctly detect the rDNA copy number, b) could be used as species-specific markers and c) might help in understanding the genetic diversity, genome organization and evolution of this species.

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## 1. Introduction

The genus *Agave* contains more than 200 species and 47 infraspecific categories; 186 taxa are distributed in Mexico, as it is the center of origin of this genus [1]. Many of these species are of great economic importance due to its high sugar content, fiber and bioactive natural products. Some agaves are mainly used to produce alcoholic beverages (Tequila, and Mezcal) [2]. Natural mead or juices obtained from cores or “piñas”, fresh or cooked, can be used to obtain polysaccharides, agave fructans, high fructose syrup, biofuel or Maillard compounds. In addition, recent attention has been focused on the potential of *Agave* species as bioenergy crops, as these plants offer many advantages for this purpose [3].

The blue agave, *Agave tequilana* Weber ‘Azul’ named by German botanist Weber, is the only variety legally permitted for the production of Tequila by the Mexican government and it has been vegetatively

spread throughout the last 200 years [4]. In economic terms for Mexico, it represents \$1.7 billion in annual revenue only within the United States [5]. In addition, this species is the most promising for bioenergy production because of its productivity, established agricultural practices, and ethanol conversion technologies [6]. *A. tequilana* is commercially propagated by asexual rhizomatous shoots, a procedure which allows for the increase of genetically elite clones with remarkable qualities (higher sugar content, size and rapid growth); however, and as a consequence, genetic diversity has been reduced though it has been subject to major disease and insect pressure [2,7]. In this context, the economic importance and vulnerability of *Agave* crops have led to an increased interest in genetic research of the species [4,8,9]. Recent studies have described a specific relationship between ploidy level, genome sizes, and the number of loci for rDNAs and repetitive sequences [10,11,12,13]; it has also showed that variation in size and structural rearrangements of the genome have meaning adaptive and influences the phenotype of two ways, both in the expression of their gene content and the physical effects of their mass or volume [10,14]. Therefore, morphological and genetic changes boost the possibility of selecting biotypes elite with useful features that can be used in breeding programs and biotechnology.

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Nuclear ribosomal DNA (rDNA) encoding 45S and 5S rRNAs belong to the most important housekeeping genes, and play a central role in cell metabolism and genome organization [15,16]. In plant genomes there may be from several hundred up to tens of thousands of highly homogeneous copies of each gene. A high copy number of these genes is probably important so the increased demand for proteosynthesis during plant development and stabilization of the cell nucleus can be ensured [17,18]. Each 45S rDNA unit consists of three coding regions (5.8S, 18S, 25S/26S in plants, 28S in animals), the internal transcribed spacers, and the intergenic spacer, which separates transcribed units [19]. The 45S units are organized in tandem arrays at one or several loci. The 5S rDNA encoding a 120-bp-long transcript has been traditionally considered to inhabit separate chromosomal locations in plants [20]; however, the 5S rDNA array may be found at more than one locus, either on the same chromosome as the 45S repeats or scattered across the genome. The rDNA arrays display substantial copy number variation within and between species [21,22,23]; this variation is functionally relevant, as it modifies chromatin states and gene expression across the genome. Moreover, the rDNA copy number has a functional significance to our understanding of crop plant domestication, and agricultural improvement as well [24].

Therefore, it is not surprising that a wide spectrum of laboratory methods has been developed to identify these rDNA copy number changes [25,26,27]. Each method is characterized by particular (dis)advantages, being the choice of a given technique largely dependent on the application, required resolution, flexibility, workload, and cost. Conventional karyotyping allows detecting structural variations across the entire genome, but it is limited in resolution (>5–10 Mb) [28]. FISH analysis for targeted regions has been used in a routine setting for many years, and requires either metaphase chromosomes (similar to karyotyping) or interphase nuclei. However, this method can be more difficult to analyze, especially when attempting to resolve tandem duplications [29]. Quantitative PCR (qPCR) technology offers fast and reliable quantification of any target sequence in a sample [30]. It also has many advantages over alternative methods, such as low consumable and instrumentation costs, fast assay development time, and high sensitivity. Recent studies have used this method for determining the rDNA copy number per genome for a better understanding of the genome organization in different species [15,22,31,32,33,34,35]. Furthermore, the studies provide evidence of a strong relationship between genome size (DNA C-value) and the rDNA copy number.

Although the current and potential economic importance of *Agaves* is irrefutable, even surprisingly, relatively little research has been carried out on these species, especially in the study of rDNA copy number variations into the genome. In this sense, more recent studies only identify the number and chromosomal location of rDNA sites in three different species of the genus *Agave*, including *A. tequilana* Weber by FISH [36]. Furthermore, Tamayo-Ordoñez et al. [37] defined the rDNA copy number using a complex method, which is based on isolation of cell nuclei and analysis of fragments by quantitative capillary electrophoresis.

The aim of this study was to develop a methodological tool based on qPCR in order to determine the copy number of the ribosomal DNA (18S and 5S) in *A. tequilana*, as well as their contribution to genome size. The results showed a variation conserved in the rDNA copy number per genome and physical distribution of the loci. This method was a contribution to the understanding of genome organization in the *Agave* species.

## 2. Materials and methods

### 2.1. Plant material

*A. tequilana* plants were grown under greenhouse conditions at day/night temperature regime of 30/25°C in the Regional Botanical

Garden of the Centre for Scientific Research of Yucatán, México (CICY). The leaves were dissected, frozen in liquid nitrogen and kept at -80°C until analysis.

### 2.2. Molecular analysis

Total DNA (gDNA) was extracted from 100 mg of leaf samples using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions and treated with RNase A (Boehringer Mannheim). The quality and quantity of the DNA were assessed spectrophotometrically (NanoDrop 2000; Thermo Scientific) by a standard procedure. In order to verify DNA integrity, extracts were fractionated by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light (High Performance UV Transilluminator, UVP).

Partial sequences of 18S and 5S rDNAs were obtained by PCR using conserved primers pairs. These primers were designed with Primer Premier v5 software (PrimerBioSoft) and conserved regions of sequences reported from other plant species (Table 1). PCR was performed in a Veriti Thermal Cycler (Applied Biosystem) and using *Taq* DNA polymerase (Invitrogen) according to the manufacturer's protocol. The PCR mix contained 100 ng DNA and 1 µL of each primer 10 µM, in 50 µL total volume. The cycling parameters were: 94°C (3 min); 35 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s); 72°C (10 min). Reaction products were separated by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide. PCR products were carefully excised and purified with a Nucleo Spin Extract Kit (Macherey-Nagel), linked to the pGEM-T Easy vector (Promega) and sequenced by Langebio (CINVESTAV, Mexico). Sequence analysis was carried out at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>) using the BlastN service. The phylogenetic tree was constructed using MEGA6 software [38] from the ClustalW alignment and the neighbor-joining method. Reliability of each node was established by the bootstrap method.

### 2.3. Quantitative PCR

Quantitative PCR (qPCR) was used to determine the copy number of 18S and 5S rDNAs. qPCR reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems) and PCR products were analyzed by 1means of StepOne Software v2.3 (Applied Biosystems). Each amplification reaction contained 12.5 µL of SYBR® Green PCR Master Mix (Applied Biosystems), 10 ng gDNA and 1 µL of each primers 1 µM, in 25 µL total volume. Cycling parameters were: 10 min at 95°C; 40 cycles of 95°C (15 s), 60°C (1 min); and melt-curve analysis.

All primers pairs gave a single peak of dissociation in all reactions, and no amplification occurred in reactions without template. PCR efficiencies for each primers pair were determined from the standard curve ( $R^2 > 0.996$  for all primers and efficiencies 104–110%). Primer sequences used for qPCR are reported in Table 1.

Known concentrations of purified plasmids (100, 10, 1, 0.1,  $1 \times 10^{-2}$ ,  $1 \times 10^{-3}$ ,  $1 \times 10^{-4}$  ng/µL) were used to develop standard curves for absolute quantification of the copy number of target genes. Standard curve was developed by plotting Ct values against Log10 values of plasmid copy numbers used as template [39]. Three independent assays were carried out. For estimation of plasmid copy number, [Equation 1] was used.

$$m = [n] \left[ \frac{1 \text{ mol}^L}{6.023 \times 10^{23} \text{ (bp)}} \right] \left[ \frac{600 \text{ g}}{\text{mol}^L} \right] \\ = [n] \left[ 1.096 \times 10^{-21} \text{ g}_{/\text{bp}} \right] \quad \text{[Equation 1]}$$

Where n = nucleotide size; m = mass; Avogadro number =  $6.023 \times 10^{23}$  molecules/mol; and average MW of a dsDNA molecule = 660 g/mol. Thus, plasmid copy number = plasmid concentration/m. From the standard curve, a regression equation was developed to obtain the

**Table 1**

Primer sequences used for PCR and qPCR, amplicon size, annealing temperature, accession number and efficiency.

Gene	Forward 5'–3'	Reverse 5'–3'	Size (bp)	Temperature (°C)	Accession number	Efficiency %
<i>PCR</i>						
18S	GCTGGAATTACCGCGGCT	CGGCTACCACATCCAAGGAA	186	60		
5S-ITS	GATCCCATCAGAACTCCG	ATGCAACACGAGGACTTCCCAG	593	60		
<i>qPCR</i>						
18S	GCTGGAATTACCGCGGCT	CGGCTACCACATCCAAGGAA	186	60	GQ983553	110
5S	CGATCATACCAGCACTAAAGCACC	ATGCAACACGAGGACTTCCCAG	111	60	GQ983554	104

slope (b) and y intercept (a). Absolute copy number (CN) in each sample was estimated by using regression equation,  $CN = \text{antilog}_{10}(Ct - a) / b$ . Lastly, rDNA number of copies per genome was determined in relation to the 4C DNA content in *A. tequilana* [37].

#### 2.4. Probes and FISH

Chromosome preparation and FISH were performed according to Robert et al. [13]. In short, Metaphase chromosomes were obtained from root tips, which were treated with paradichlorobenzene (PDB) at 25°C for 3 h, fixed in 3:1 ethanol-glacial acetic acid for 12 h, and then transferred to 70% ethanol at -20°C until use. Root tips were washed in standard saline citrate buffer (SSC) and digested with 50% (v/v) of Viscozyme® L (Sigma V2010) at 37°C for 25 min. Meristematic cells were dissected, gently pipetted onto slides, macerated in a drop of a 60% acetic acid, and squashed with a glass coverslip. Slides were frozen on liquid nitrogen and the cover slip was flipped off with razor blade. The 18S probe includes a 1 kb fragment isolated from *Triticum aestivum*, which was labeled with digoxigenin-11-dUTP by Nick translation and detected with anti-digoxigenin-fluorescein isothiocyanate (FITC). A probe for 5S sequences rDNA sequences was prepared by amplifying and labeling the 120 bp genic sequence from *Nicotiana rustica*, in the presence of biotin-16-dUTP. Biotin-labeled probes were detected with avidin-cyanine-3 (Cy3).

For FISH, the probe mixture was prepared as described by Robert et al. [13]. After overnight hybridization at 37°C, slides were given a stringent wash in 20% (v/v) formamide in 0.1% SSC at 40–42°C. Sites of probe hybridization were detected using 20 mg mL<sup>-1</sup> Anti-Digoxigenin-Fluorescein (Roche Biochemicals) and 5 mg mL<sup>-1</sup> Cy3-conjugated avidin (Amersham Pharmacia) in 4% SSC containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin. Chromosomes were counterstained with 2 mg mL<sup>-1</sup> 4', 6-diamidino-2-phenylindole (DAPI) in 4% SSC, mounted in Vectashield (Vector Laboratories) medium, and examined using a Leica DMRA2 epifluorescent microscope fitted with an Orca ER camera and Open Lab software (Improvision). All images were processed with Adobe Photoshop, treated with a uniform color contrast and brightness. For all the probes, three to ten metaphase cells were analyzed. Densitometric units (DU) of the ribosomal DNA images were estimated using ImageJ software [40].

### 3. Results and discussion

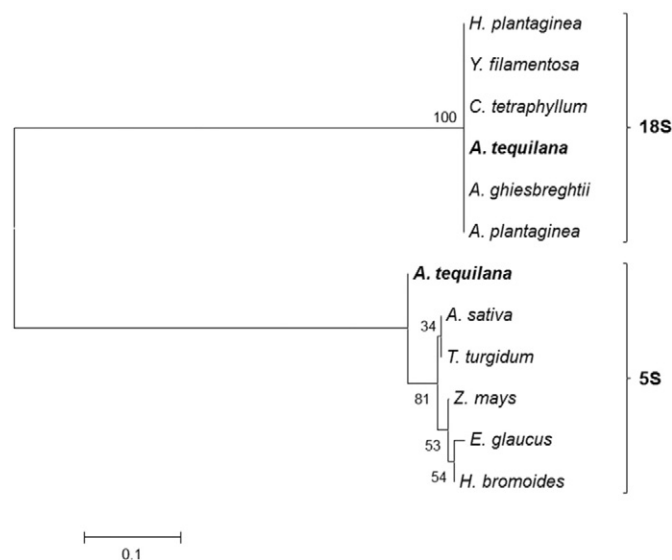
#### 3.1. Cloning and sequences analysis of ribosomal DNA

Based on the highly conserved sequence of rDNA genes of plants, a DNA fragment of 18S rDNA and complete 5S rDNA were initially isolated with specific primers by PCR from *A. tequilana* leaves (Table 1). BLAST and phylogenetic analysis confirmed the close sequence homology of these amplicons with the corresponding genes in different plant species (Fig. 1). 18S rDNA (186 bp) show 100% identity with *Agave ghiesbreghtii*, including the most representative kinds of the Agavaceae subfamily (*Anthericum*, *Chlorophytum*, *Hosta*, *Yucca*) [41]. The 18S regions have remained relatively constant within the same family (Asparagaceae), making these DNA regions the important of information for the study of phylogenetic relationships

[42]. On the other hand, a search of public databases (GenBank/EMBL/DBJ) showed that to date no data of 5S-ITS sequences for the Asparagaceae family were found; therefore, we were unable to compare the sequences in this family. In spite of this, 5S rDNA (505 bp) had a 97–98% homology with various species of the Poaceae family, showing that the 5S sequence of *A. tequilana* has conserved regions with species of economic importance (eg. *Avena sativa*, *Triticum turgidum* and *Zea mays*). The complete sequence includes a conserved region (121 bp) and internal transcribed spacers (384 bp). In eukaryotic cells, the 5S rDNA encoding a 120-bp-long transcript with a molecular mass of 40 kDa [43]. The secondary and tertiary structures are generally conserved across phylogeny [20,44]. In both cases, partial or complete sequences of individual rDNA indicate the high evolutionary conservation of the amplified fragments.

#### 3.2. Generation of standard curve and quantification assay

The establishment of a standard curve using the qPCR process is one of the key steps in determining the copy number of a given target sequence. Standard curves for the 18S and 5S rDNAs were generated from known starting pDNA concentrations, and used to determine the copy number per genome of these ribosomal genes in an amount DNA sample by interpolation. To generate the curve, amplifications on serial dilutions of the plasmid containing the 18S and 5S rDNA fragments were performed and a linear relationship between the level of fluorescence ( $\Delta Ct$ ) and the input amount of pDNA was produced (Fig. 2). The  $\Delta Ct$  values were



**Fig. 1.** Phylogenetic tree of the 18S and 5S rDNA of *A. tequilana* and other species. This was inferred using the Neighbor-Joining method (bootstrap test 1000) in the MEGA 6 package. The numbers at the nodes are the percentage bootstrap values. The scale bar represents genetic distance. The GenBank accession numbers used in this phylogenetic analysis were as follows: *A. tequilana* 18S (GQ983553); *A. ghiesbreghtii* (AF206841); *C. tetraphyllum* (HM640724); *A. liliago* (HM640720); *Y. filamentosa* (HM640713); *H. plantaginea* (HM640711); *A. tequilana* 5S (GQ983554); *A. sativa* (EF071691); *Z. mays* (DQ351339); *H. bromoides* (AJ390159); *T. turgidum* (FJ882491); *E. glaucus* (KJ793289).

then plotted against the log of the initial copy number of template pDNA, ranging from  $1 \times 10^3$  to  $1 \times 10^{10}$  copies/ $\mu\text{L}$ . The correlation ( $R$  [2]) between  $\Delta\text{Ct}$  value and log pDNA concentration was  $>0.996$  for the 18S and 5S rDNAs. The slopes ( $-3.238$  and  $-3.095$ ) of the regression equation indicated very efficient amplification rates (Table 1). This demonstrates that there is a high correlation between the original amount of pDNA in the template and the Ct value obtained after amplification as well as a high efficiency of the qPCR reaction [30,45,46]. The absolute quantification method relies on a standard plot constructed from known concentrations of standards to measure the actual copy numbers of a particular target, and is therefore considered to be more informative and reliable for comparisons [47,48]. The quantity of target in samples can be evaluated with reasonable accuracy only by using a properly characterized standard.

### 3.3. Determination of ribosomal DNA copy number in *A. tequilana*

Estimation of copy number of 18S and 5S rDNAs was determined with a fixed amount of total DNA (10 ng) from five different plants of *A. tequilana*. Absolute quantification was performed using the  $\Delta\text{Ct}$  values and the standard curves were constructed. Comparison of  $\Delta\text{Ct}$  values for each sample shows minimal variation based on standard deviation and coefficient of variance (Fig. 3; Table 2). This demonstrates the

reproducibility and the value in the introduction of qPCR for the quantitative detection of the ribosomal genes. The mean Ct values were used to determine the copy number per genome and their contribution to genome size of *Agave* (Table 2). Copy number was based on the 4C DNA content (15.01 pg) of the *A. tequilana* genome [13]. The number of copies of the 18S rDNA was 51 times higher than the 5S rDNA, contributing to genome size of 1.47 and  $8.38 \times 10^{-3}\%$ , respectively. This result is in contrast to previous results, which have reported a greater number of copies per genome of the 5S rDNA, with 1.5 and 1.92 times more than the 18S rDNA in *A. tequilana* and species of the genus *Agave*, respectively [37]. This difference may be because the rDNA copy number was determined by using quantitative capillary electrophoresis, in relation to the area under each peak (fluorescence intensity) of the different sizes for each rDNA, as well as the variation of the amount of DNA estimated in about 30,000 nuclei  $\cdot\text{mL}^{-1}$ , for each *Agave* L. accession [37,49]. In general, these results showed a wide range of variation in rDNA gene proportion for each species tested. Meanwhile, Gomez-Rodriguez et al. [36] performed the physical mapping of 5S and 18S ribosomal DNA in three species of *Agave*, but this method does not have the resolution ( $>1$  kb or tandem sequences) to determine the number of copies [25]. At present, qPCR analysis allows to determine polymorphisms single nucleotide (SNP), small insertion-deletion, also genomic variation between species, with a detection capability of the

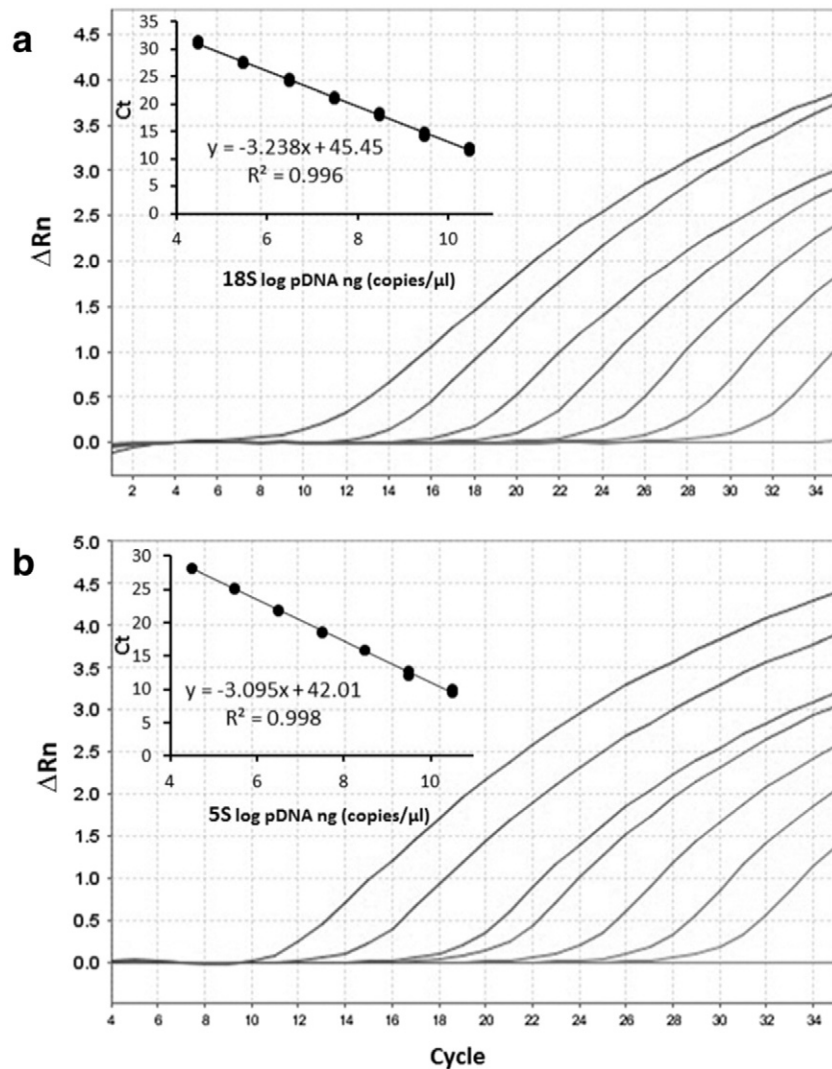
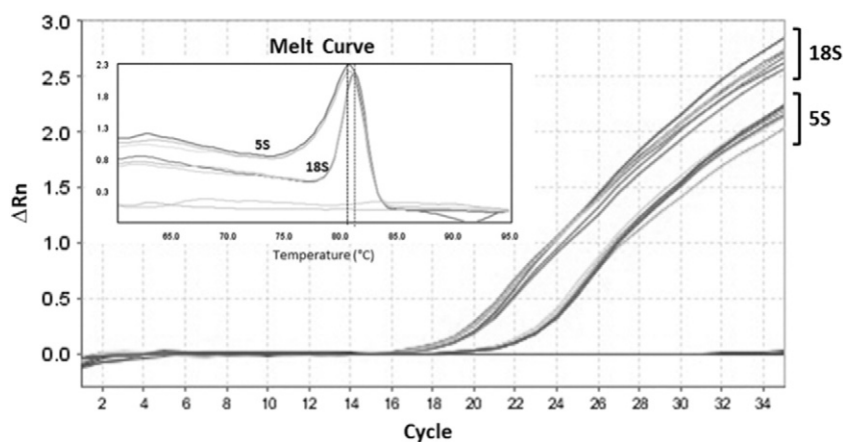


Fig. 2. qPCR detections show the development of fluorescence signal of the serial dilutions of the pDNA of 18S (a) and 5S (b) rDNA. Amplification plots were generated by serial dilutions of pDNA ranging from 100 ng to 0.1 pg and specific primer pairs. In each case, the standard curves were generated from amplification data. The reproducibility was analyzed by evaluating the slope and correlation coefficient of the curve. Three replicates were performed for each sample.





**Fig. 3.** Amplification plot of 18S and 5S rDNA using genomic DNA (10 ng) from five different plants of *A. tequilana*. Melt curve analysis, the change in fluorescence intensity is plotted in function of temperature. Three replicates were performed for each sample.

copy number in a range of 1 to 4 in a diploid genome [25,29]. The qPCR technique offers great flexibility and adaptability, and can be carried out in a closed system, thus eliminating the risk of PCR and sample contamination and does not require postprocessing of PCR products. Consequently, the estimate of copy number of rDNAs and DNA transposons in the genome of different organisms (plants, algae, fungus, and animals) has been determined based on the application of qPCR [12,22,23,50]. In this regard, application of qPCR, DNA C-values and analysis of target genes have been proposed as a viable methodology for estimating its contribution to the genome [12,21,35,51]. Comparison of rDNA copy number per genome from our data with those reported in plants show close relationship (150 to 75,000 rDNA copies), although copy number per genome may vary greatly within a given species [35, 52,53]. In a set of microalgae and unicellular algae, numbers range from 1 to 36,896 rDNA copies [32,34]; in fungi range from 60 to 220 [54]; and in protozoa range from 3385 to 315,786 [22]. Moreover, copy numbers in *A. tequilana* are generally higher in both animals (39–19,300) and humans (100–12,900) [34,55]. Therefore, rDNA copy number variations suggest evolutionary mechanisms (gene duplication processes, structural rearrangements and genome mutations) of these essential genes for each species [21,26], providing a mechanism for cell homeostasis and quick and reversible adaptation. Copy number variation mainly affects the members of large families of functionally redundant genes [29,31]. The effects of this variation on phenotype are usually modest; nevertheless, there are many cases in which they have been linked to important traits such as resistance to biotic and abiotic stress. In economically important plants, changes in the rDNA copy number may be related to biological processes (flowering time, plant height and response to stress), and genome organization (size, composition, ploidy level), allowing selection of plants with outstanding agronomic characteristics [56].

### 3.4. Comparison of FISH images and the rDNA copy number by qPCR

In order to confirm the copy number of 18S and 5S rDNAs in the genome of *A. tequilana*, physical mapping of rDNAs was examined using dual color fluorescence *in situ* hybridization FISH (Fig. 4). The diploid chromosome number was confirmed  $2n = 60$ , with bimodal karyotype composed of 5 pairs of large chromosomes and 25 pairs of small chromosomes [13,36,57]. The 18S rDNA was associated with the secondary constriction of a large chromosome pair, while 5S rDNA was located in both arms of a small chromosome pair. Also, the 18S rDNA signals were higher than those observed for 5S rDNA signals. Comparing the signals obtained for 18s ( $151,992 \pm 109$  DU) and 5s ( $36,539 \pm 3721$  DU) rDNAs with copy number per genome of *A. tequilana* showed a linear relationship ( $R^2 = 0.992$ ) and evidenced that qPCR estimates of the different rDNAs were very coherent with FISH data. This approach has been used in the identification and characterization of rDNA copy number variations in genome of plants and microalgae [29,34], and it establishes a good relationship between rDNA copies and direct visualization of DNA copy number at the level of individual cells. It also suggests that the qPCR data could be used as a better analysis when attempting to resolve tandem duplications, since FISH is considered a semi-quantitative technique [58], because the size and intensity of hybridization signals are an indicator of targeted sequence copy number. On the other hand, *Agave* genus exhibits different ploidy levels ranging from diploid (*A. tequilana*,  $2x$ ) to octaploid ( $8x$ ) [1,4,11,13], regardless of polyploidy, the bimodal karyotypes are maintained, and suggests that the process of speciation in *Agave* was due to structural rearrangements in chromosome groups [1,58]. However, complex interactions between these factors and environmental growing conditions exist [10], which have resulted in the loss and gain of rDNA loci and probably also in copy repeat

**Table 2**

Determination of copy number of the 18S and 5S rDNAs in the genome of *Agave tequilana* plant.

Genes	gDNA (ng)	$\Delta Ct^a$	$CV^b$	Copies/ng <sup>c</sup>	Copy number per genome <sup>d</sup>	Copy number per haploid genome	Reported size gene (bp)	Gene size per genome (pg) <sup>e</sup>	Genome size contribution (%) <sup>f</sup>
18S	10	$19.4 \pm 0.29$	0.014	8,332,729	124,905	62,453	1741 (AF206876)	0.22	1.47
5S	10	$22.7 \pm 0.27$	0.012	165,955	2442	1221	505 (GQ983554)	$1.25 \times 10^{-3}$	$8.38 \times 10^{-3}$

1 pg of DNA =  $9.80 \times 10^8$  base pairs (bp), Bennett and Leitch [59].

<sup>a</sup>  $\Delta Ct$  average of five individuals with three independent repetitions.

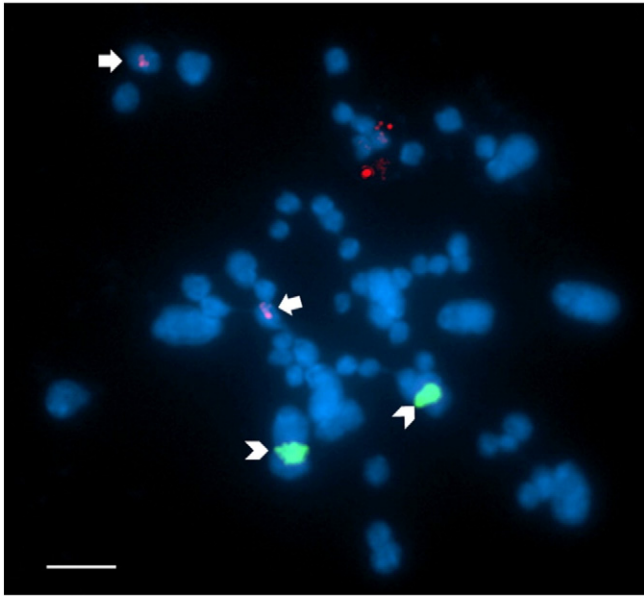
<sup>b</sup> Coefficient of variance.

<sup>c</sup> Copy number calculated with the equation of the standard curve.

<sup>d</sup> Copy number per genome = [copies/ng] [15.01 pg / 1000]; *A. tequilana* Weber "Azul" ploidy level ( $2n = 2x = 60$ ), 4C DNA content 15.01 pg, Robert et al. [13].

<sup>e</sup> Gene size per genome (pg) = [copy number per genome] [complete sequence rDNA (bp)] /  $9.80 \times 10^8$  (bp).

<sup>f</sup> Genome size contribution = [gene size per genome (pg)] [0.1501].



**Fig. 4.** Fluorescent *in situ* hybridization (FISH) of 18S and 5S rDNA in root tip metaphase of *A. tequilana*. Two hybridization sites of 18S rDNA (green and arrowheads) and 5S rDNA (pink, arrows). Scale bar, 10  $\mu$ m.

number variations in each rDNA arrays. In some economically important crops such as banana, wheat and potato, these variations affect the copy number and the expression level of polymorphism in the rDNA, increasing the occurrence of productive traits in these crops [10,56]. Therefore, this method could be used to study repeated regions (rDNA) into the genome, not only *A. tequilana* if not in this kind, helping us to understand some of the processes in the genome organization, genetic diversity and adaptive plasticity of these species.

#### 4. Conclusions

In this current study the methodology for estimating the copy number of 18s and 5s rDNAs and the contribution of these to the genome size of *A. tequilana* are reported. Our estimate is based on the generation of standard curves using the absolute quantification by qPCR and 4C DNA content reported for this species. This methodology happened to be fast and robust, validating variability using different concentrations of pDNA and gDNA from different *A. tequilana* plants. The resulting dataset proved that the copy number of 18s was greater than 5s per genome, as well as the percentage of genome size contribution. These data were confirmed through FISH and presented a significant correlation between both analyzes. The association between rDNA copy number and genome size provides convincing evidence of a strong relationship between the two and among different groups of organism. However, more research must be conducted to understand the genetic changes that influence the genome organization in *Agave* species and develop to breeding programs to preserve biodiversity and the use of biotypes elite with desirable production traits.

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#### Conflict of interest

There is no conflict of interest.

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#### References

- [1] Narváez-Zapata JA, Sánchez-Teyer LF. Agaves as a raw material: Recent technologies and applications. *Recent Pat Biotechnol* 2009;3:185–91. <http://dx.doi.org/10.2174/187220809789389144>.
- [2] Ramírez-Malagón R, Borodanenko A, Pérez-Moreno L, Salas-Araiza MD, Nuñez-Palenius H, Ochoa-Alejo N. *In vitro* propagation of three *Agave* species used for liquor distillation and three for landscape. *Plant Cell Tissue Organ Cult* 2008;94:201–7. <http://dx.doi.org/10.1007/s11240-008-9405-x>.
- [3] Davis SC, Dohleman FG, Long SP. The global potential for *Agave* as a biofuel feedstock. *GCB Bioenergy* 2011;3:68–78. <http://dx.doi.org/10.1111/j.1757-1707.2010.01077.x>.
- [4] Simpson J, Martínez Hernández A, Abraham Juárez M, Delgado Sandoval S, Sánchez Villarreal A, Cortés Romero C. Genomic resources and transcriptome mining in *Agave tequilana*. *GCB Bioenergy* 2011;3:25–36. <http://dx.doi.org/10.1111/j.1757-1707.2010.01079.x>.
- [5] Distilled spirits council of the United States: U.S. tequila market at a glance. Washington, D.C.: Distilled Spirits Council of the United States; 2011 [http://www.discus.org/assets/1/7/DISCUS\\_2011\\_Industry\\_Review\\_Final.pdf](http://www.discus.org/assets/1/7/DISCUS_2011_Industry_Review_Final.pdf).
- [6] Huitrón C, Pérez R, Gutiérrez L, Lappe P, Petrosyan P, Villegas J, et al. Bioconversion of *Agave tequilana* fructans by exo-inulinases from indigenous *Aspergillus niger* CH-A-2010 enhances ethanol production from raw *Agave tequilana* juice. *J Ind Microbiol Biotechnol* 2013;40:123–32. <http://dx.doi.org/10.1007/s10295-012-1211-0>.
- [7] Rodríguez-Sahagún A, Acevedo-Hernández G, Rodríguez-Domínguez JM, Rodríguez-Garay B, Cervantes-Martínez J, Castellanos-Hernández OA. Effect of light quality and culture medium on somatic embryogenesis of *Agave tequilana* Weber var. Azul. *Plant Cell Tissue Organ Cult* 2011;104:271–5. <http://dx.doi.org/10.1007/s11240-010-9815-4>.
- [8] Gross SM, Martin JA, Simpson J, Abraham-Juarez MJ, Wang Z, Visel A. *De novo* transcriptome assembly of drought tolerant CAM plants, *Agave deserti* and *Agave tequilana*. *BMC Genomics* 2013;14:563. <http://dx.doi.org/10.1186/1471-2164-14-563>.
- [9] Díaz-Martínez M, Nava-Cedillo A, Guzmán-López JA, Escobar-Guzmán R, Simpson J. Polymorphism and methylation patterns in *Agave tequilana* Weber var. 'Azul' plants propagated asexually by three different methods. *Plant Sci* 2012;185:6:321–30. <http://dx.doi.org/10.1016/j.plantsci.2012.01.004>.
- [10] Tamayo-Ordóñez MC, Espinosa-Barrera LA, Tamayo-Ordóñez YJ, Ayil-Gutiérrez B, Sánchez-Teyer LF. Advances and perspectives in the generation of polyploid plant species. *Euphytica* 2016;209:1–22. <http://dx.doi.org/10.1007/s10681-016-1646-x>.
- [11] Palomino G, Martínez J, Cepeda-Cornejo V, Pimienta-Barrios E. Nuclear genome size and cytotype analysis in *Agave cupreata* Trel. & Berger (Agavaceae). *Caryologia* 2012; 65:281–94. <http://dx.doi.org/10.1080/00087114.2012.752915>.
- [12] Khaliq I, Khan MA, Pearce S. Ty1-Copia retrotransposons are heterogeneous, extremely high copy number and are major players in the genome organization and evolution of *Agave tequilana*. *Genet Resour Crop Evol* 2012;59:575–87. <http://dx.doi.org/10.1007/s10722-011-9705-6>.
- [13] Robert ML, Lim KY, Hanson L, Sánchez-Teyer F, Bennett MD, Leitch RA, et al. Wild and agronomically important *Agave* species (Asparagaceae) show proportional increases in chromosome number, genome size, and genetic markers with increasing ploidy. *Bot J Linn Soc* 2008;158:215–22. <http://dx.doi.org/10.1111/j.1095-8339.2008.00831.x>.
- [14] De la Peña C, Nic-Cán G, Ojeda G, Herrera-Herrera JL, López-Torres A, Wrobel K, et al. *KNOX1* is expressed and epigenetically regulated during *in vitro* conditions in *Agave* spp. *BMC Plant Biol* 2012;12:203. <http://dx.doi.org/10.1186/1471-2229-12-203>.
- [15] Malone JH. Balancing copy number in ribosomal DNA. *Proc Natl Acad Sci U S A* 2015; 112:2635–6. <http://dx.doi.org/10.1073/pnas.1500054112>.
- [16] Tosto D, Hopp E. Characterization of the nuclear ribosomal DNA unit in *Oxalis tuberosa* (Oxalidaceae) and related species. *Electron J Biotechnol* 2008;11:1–17. <http://dx.doi.org/10.2225/vol11-issue3-fulltext-1>.
- [17] Zhang L, Hu Y, Yan S, Li H, He S, Huang M, Li L. ABA-mediated inhibition of seed germination is associated with ribosomal DNA chromatin condensation, decreased transcription, and ribosomal RNA gene hypocacetylation. *Plant Mol Biol* 2012;79: 285–93. <http://dx.doi.org/10.1007/s11103-012-9912-3>.
- [18] Wicke S, Costa A, Muñoz J, Quandt D. Restless 5S: The re-arrangement(s) and evolution of the nuclear ribosomal DNA in land plants. *Mol Phylogenet Evol* 2011;61: 321–32. <http://dx.doi.org/10.1016/j.ympev.2011.06.023>.
- [19] Wang Y, Tian RM, Gao ZM, Bougouffa S, Qian PY. Optimal eukaryotic 18S and universal 16S/18S ribosomal RNA primers and their application in a study of symbiosis. *PLoS One* 2014;9:e90053. <http://dx.doi.org/10.1371/journal.pone.0090053>.
- [20] Ciganda M, Williams N. Eukaryotic 5S rRNA biogenesis. *Wiley Interdiscip Rev RNA* 2011;2:523–33. <http://dx.doi.org/10.1002/wrna.74>.
- [21] Gibbons JG, Branco AT, Godinho SA, Yu S, Lemos B. Concerted copy number variation balances ribosomal DNA dosage in human and mouse genomes. *Proc Natl Acad Sci U S A* 2015;112:2485–90. <http://dx.doi.org/10.1073/pnas.1416878112>.
- [22] Gong J, Dong J, Liu X, Massana R. Extremely high copy numbers and polymorphisms of the rDNA operon estimated from single cell analysis of oligotrich and peritrich ciliates. *Protist* 2013;164:369–79. <http://dx.doi.org/10.1016/j.protis.2012.11.006>.
- [23] Eagle SH, Crease TJ. Copy number variation of ribosomal DNA and *Pokey* transposons in natural populations of *Daphnia*. *Mobile DNA* 2012;3:4. <http://dx.doi.org/10.1186/1759-8753-3-4>.

- [24] Matyášek R, Renny-Byfield S, Fulneček J, Macas J, Grandbastien MA, Nichols R, et al. Next generation sequencing analysis reveals a relationship between rDNA unit diversity and locus number in *Nicotiana* diploids. *BMC Genomics* 2012;13:722. <http://dx.doi.org/10.1186/1471-2164-13-722>.
- [25] Ceulemans S, van der Ven K, Del-Favero J. Targeted screening and validation of copy number variations. *Methods Mol Biol* 2012;838:311–28. [http://dx.doi.org/10.1007/978-1-61779-507-7\\_15](http://dx.doi.org/10.1007/978-1-61779-507-7_15).
- [26] Gibbons JG, Branco AT, Yu S, Lemos B. Ribosomal DNA copy number is coupled with gene expression variation and mitochondrial abundance in humans. *Nat Commun* 2014;5:4850. <http://dx.doi.org/10.1038/ncomms5850>.
- [27] Garcia S, Kovařík A. Dancing together and separate again: Gymnosperms exhibit frequent changes of fundamental 5S and 35S rRNA gene (rDNA) organisation. *Heredity* 2013;111:23–33. <http://dx.doi.org/10.1038/hdy.2013.11>.
- [28] Cantalieris S, Baird PN, White SJ. Molecular methods for genotyping complex copy number polymorphisms. *Genomics* 2013;101:86–93. <http://dx.doi.org/10.1016/j.ygeno.2012.10.004>.
- [29] Zmieňko A, Samelak A, Kozłowski P, Figlerowicz M. Copy number polymorphism in plant genomes. *Theor Appl Genet* 2014;127:1–18. <http://dx.doi.org/10.1007/s00122-013-2177-7>.
- [30] Gambetta GA, McElrone AJ, Matthews MA. Genomic DNA-based absolute quantification of gene expression in *Vitis*. *Physiol Plant* 2013;148:334–43. <http://dx.doi.org/10.1111/pp1.12014>.
- [31] Renny-Byfield S, Kovarik A, Kelly LJ, Macas J, Novak P, Chase MW, et al. Diploidization and genome size change in allopolyploids is associated with differential dynamics of low- and high-copy sequences. *Plant J* 2013;74:829–39. <http://dx.doi.org/10.1111/tpj.12168>.
- [32] Godhe A, Asplund ME, Härnström K, Saravanan V, Tyagi A, Karunasagar I. Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by Real-Time PCR. *Appl Environ Microbiol* 2008;74:7174–82. <http://dx.doi.org/10.1128/AEM.01298-08>.
- [33] Richard GF, Kerrest A, Dujon B. Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. *Microbiol Mol Biol Rev* 2008;72:686–727. <http://dx.doi.org/10.1128/MMBR.00011-08>.
- [34] Zhu F, Massana R, Not F, Marie D, Vaulot D. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. *FEMS Microbiol Ecol* 2005;52:79–92. <http://dx.doi.org/10.1016/j.femsec.2004.10.006>.
- [35] Prokopowich CD, Gregory TR, Crease TJ. The correlation between rDNA copy number and genome size in eukaryotes. *Genome* 2003;46:48–50. <http://dx.doi.org/10.1139/G02-103>.
- [36] Gomez-Rodriguez VM, Rodriguez-Garay B, Palomino G, Martínez J, Barba-Gonzalez R. Physical mapping of 5S and 18S ribosomal DNA in three species of *Agave* (Asparagales, Asparagaceae). *Comp Cytogenet* 2013;7:191–203. <http://dx.doi.org/10.3897/CompCytogen.v7i3.5337>.
- [37] Tamayo-Ordóñez YJ, Narváez-Zapata JA, Sánchez-Teyer LF. Comparative characterization of ribosomal DNA regions in different *Agave* accessions with economical importance. *Plant Mol Biol Rep* 2015;33:1–16. <http://dx.doi.org/10.1007/s11105-015-0895-5>.
- [38] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;30:2725–9. <http://dx.doi.org/10.1093/molbev/mst197>.
- [39] Dhanasekaran S, Doherty TM, Kenneth J. Comparison of different standards for real-time PCR-based absolute quantification. *J Immunol Methods* 2010;354:34–9. <http://dx.doi.org/10.1016/j.jim.2010.01.004>.
- [40] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671–5. <http://dx.doi.org/10.1038/nmeth.2089>.
- [41] McKain MR, Wickett N, Zhang Y, Ayyampalayam S, McCombie WR, Chase MW, et al. Phylogenomic analysis of transcriptome data elucidates co-occurrence of a paleopolyploid event and the origin of bimodal karyotypes in Agavoideae (Asparagaceae). *Am J Bot* 2012;99:397–406. <http://dx.doi.org/10.3732/ajb.1100537>.
- [42] Angiosperm Phylogeny Group. An update of the Angiosperm Phylogeny group classification for the orders and families of flowering plants: APG III. *Bot J Linn Soc* 2009;161:105–21. <http://dx.doi.org/10.1111/j.1095-8339.2009.00996.x>.
- [43] Garcia S, Crhák Khaitová L, Kovařík A. Expression of 5S rRNA genes linked to 35S rDNA in plants, their epigenetic modification and regulatory element divergence. *BMC Plant Biol* 2012;12:95. <http://dx.doi.org/10.1186/1471-2229-12-95>.
- [44] Li L, Cai-Hong Z, Yin-Bing B. The molecular diversity analysis of *Auricularia auricula-judae* in China by nuclear ribosomal DNA intergenic spacer. *Electron J Biotechnol* 2014;17:27–33. <http://dx.doi.org/10.1016/j.ejbt.2013.12.005>.
- [45] Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. Multiplex qPCR for detection and absolute quantification of malaria. *PLoS One* 2013;8, e71539. <http://dx.doi.org/10.1371/journal.pone.0071539>.
- [46] Corradi N, Croll D, Colard A, Kuhn G, Ehinger M, Sanders IR. Gene copy number polymorphisms in an arbuscular mycorrhizal fungal population. *Appl Environ Microbiol* 2007;73:366–9. <http://dx.doi.org/10.1128/AEM.01574-06>.
- [47] Shimada H, Obayashi T, Takahashi N, Matsui M, Sakamoto A. Normalization using ploidy and genomic DNA copy number allows absolute quantification of transcripts, proteins and metabolites in cells. *Plant Methods* 2010;6:29. <http://dx.doi.org/10.1186/1746-4811-6-29>.
- [48] Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *J Biotechnol* 2006;123:273–80. <http://dx.doi.org/10.1016/j.jbiotec.2005.11.014>.
- [49] D'haene B, Vandesompele J, Hellemans J. Accurate and objective copy number profiling using real-time quantitative PCR. *Methods* 2010;50:262–70. <http://dx.doi.org/10.1016/j.jymeth.2009.12.007>.
- [50] Herrera ML, Vallor AC, Gelfond JA, Patterson TF, Wickes BL. Strain-dependent variation in 18S ribosomal DNA Copy numbers in *Aspergillus fumigatus*. *J Clin Microbiol* 2009;47:1325–32. <http://dx.doi.org/10.1128/JCM.02073-08>.
- [51] Bennett MD, Leitch IJ. Nuclear DNA amounts in angiosperms: Targets, trends and tomorrow. *Ann Bot* 2011;107:467–590. <http://dx.doi.org/10.1093/aob/mcq258>.
- [52] Boutte J, Aliaga B, Lima O, Ferreira de Carvalho J, Ainouche A, Macas J, et al. Haplotype detection from next generation sequencing in high ploidy-level species: 45S rDNA gene copies in the hexaploid *Spartina maritima*. *Genes Genomes Genet* 2016;6:29–40. <http://dx.doi.org/10.1534/g3.115.023242>.
- [53] Long Q, Rabanal FA, Meng D, Huber CD, Farlow A, Platzer A, et al. Massive genomic variation and strong selection in *Arabidopsis thaliana* lines from Sweden. *Nat Genet* 2013;45:884–90. <http://dx.doi.org/10.1038/ng.2678>.
- [54] Simon UK, Weiss M. Intragenomic variation of fungal ribosomal genes is higher than previously thought. *Mol Biol Evol* 2008;25:2251–4. <http://dx.doi.org/10.1093/molbev/msn188>.
- [55] Stults DM, Killen MW, Pierce HH, Pierce AJ. Genomic architecture and inheritance of human ribosomal RNA gene clusters. *Genome Res* 2008;18:13–8. <http://dx.doi.org/10.1101/gr.6858507>.
- [56] Renny-Byfield S, Wendel JF. Doubling down on genomes: Polyploidy and crop plants. *Am J Bot* 2014;101:1711–25. <http://dx.doi.org/10.3732/ajb.1400119>.
- [57] Palomino G, Martínez J, Méndez I. Karyotype studies in cultivars of *Agave tequilana* Weber. *Caryologia* 2008;61:144–53. <http://dx.doi.org/10.1080/00087114.2008.10589622>.
- [58] Aversano R, Ercolano MR, Caruso I, Fasano C, Rosellini D, Carputo D. Molecular tools for exploring polyploid genomes in plants. *Int J Mol Sci* 2012;13:10316–35. <http://dx.doi.org/10.3390/ijms130810316>.
- [59] Bennett MD, Leitch IJ. Nuclear DNA amounts in angiosperms: Progress, problems and prospects. *Ann Bot* 2005;95:45–90. <http://dx.doi.org/10.1093/aob/mci003>.