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Research article

A quantitative PCR approach for determining the ribosomal DNA copy number in the genome of *Agave tequila* 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 8.38×10^{-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.

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 fractioned by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light (High Performance UV Transiluminator, 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, 1×10^{-2}, 1×10^{-3}, 1×10^{-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.

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

Where n = nucleotide size; m = mass; Avogadro number = 6.023×10^{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