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ORIGINAL PAPER

Comparative Characterization of Ribosomal DNA Regions in Different *Agave* Accessions with Economical Importance

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Abstract Two ribosomal DNA regions (5S and 18S) were characterized in three economically important species of *Agave* Linnaeus, 1753 namely *Agave tequilana* Weber, 1902; *Agave angustifolia* Haworth, 1915; and *Agave fourcroydes* Lemaire, 1864 which are used to produce several products such as tequila, mezcal, and hard fibers. Characterization included *Agave* L. accessions with different ploidy levels ($2n=2x=60$ to $2n=6\times=180$) in order to relate this factor with copy number, haplotype number, expression profile, and predictable functionality of ribosomal DNA (rDNA) sequences. Only total rDNA copy number (5S and 18S) was related with ploidy level. Main differences were found in the 5S rDNA gene since it exhibited different genetic traits of *Agave* L. accession. In this gene, four different allelic groups (I, 105; II, 107; III, 110; and IV, 111 bp) were detected, which have probably evolved separately, thus exhibiting different expression profiles and different haplotype occurrence. Allelic groups III and IV exhibit the highest number of total and expressed copies in all *Agave* L. accessions. Non-redundant haplotypes were probably more functional in these allelic groups. Differences between the *Agave* L. accessions were more clearly observed in the most cultivated accession, *A. tequilana* ($2n=2\times=60$), where the allelic group III shows non-redundant haplotypes and is transcriptionally upregulated suggesting a different evolutionary pressure on this *Agave* L. accession.

Keywords DNA ribosomal · *Agave* · Variability · Polyploidy · Agavaceae · Haplotypes · Functional rDNA

Introduction

Agave Linnaeus, 1753 is a genus containing more than 200 species of which 75 % are found in Mexico, which is thought to be the center of origin for this genus (Eguiarte et al. 2000; García-Mendoza 2007). The *Agave* L. genus has a recent origin about eight million years ago, with a high diversity index of 0.32 to 0.56 species per million years (Good-Avila et al. 2006). This is a high value when compared with other angiosperms (0.089 to 0.77) (Magallón and Sanderson 2001). As consequence of this diversity, new polyploidy species are currently being described which further increases the diversity of the genus (Massey and Hamrick 1998; Palomino et al. 2003). High diversity in *Agave* L. might be due to a fast speciation process, which is probably a consequence of chromosomal structural rearrangements and single and spontaneous heterozygous mutations (Castorena-Sánchez et al. 1991; Cavallini et al. 1995). This genus exhibits different ploidy levels in most of its species with levels of $2n=2\times=60$ to $2n=8\times=240$ with a constant basic chromosomic number of $n=30$ (Granick 1944; Doughty 1936; Brandham 1969; Barba-Gonzalez et al. 2013). Usually, ploidy level has been described as a value trait in some economically important crops such as banana, wheat, and potato (Feldman et al. 1997; Hawkes 1979; Thompson et al. 2004; Román et al. 2004). In these crops, polyploidy variations affect the copy number and the expression level of some important genes in comparison with their diploid

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ancestors (Kashkush et al. 2002; Dadejová et al. 2007; Kovarik et al. 2008) increasing the occurrence of productive traits in these polyploidy crops.

Agave L. polyploid species have been widely used in nutrition and medicine, for religious purposes, as textiles, in construction, and even for ornamental uses (Gentry 1982; Valenzuela 1997; Nobel 1998). *Agave tequilana* Weber, 1902; *Agave angustifolia* Haworth, 1915; and *Agave fourcroydes* Lemaire, 1864 are particularly important as they are used to produce several important products such as tequila, mezcal, and hard fibers (Robert et al. 2008; Palomino et al. 2003; Palomino et al. 2005). As consequence of the commercial importance of these species, some of them are being intensively cultivated, selecting only a limited number of productive traits (Colunga-GarcíaMarín and May-Pat 1997; Vargas-Ponce et al. 2009). Some authors have suggested that this management strategy can reduce the genetic variability in the *Agave* L. genus (Colunga-GarcíaMarín et al. 1999; Eguiarte et al. 2000). As a result, it is thought that the more intensively cultivated *A. tequilana* Weber therefore only exhibits 25 % of its genetic variability when compared to wild populations of the closely related *A. angustifolia* Haw. (Vargas-Ponce et al. 2007; Vargas-Ponce et al. 2009). One of the major challenges in cultivating this genus has been increasing its productive traits to gain commercial value without affecting its variability and genetic diversity.

In the genus *Agave* L., the ribosomal loci (5S and 45S) are present on different chromosomes, and there is an apparent constant increase in loci according to ploidy level (Robert et al. 2008). Consequently, these loci might be used as genetic markers for polyploidy events. The current study aimed to explore, in more details, the ploidy evolutionary phenomena by comparative characterization of these ribosomal DNA loci in several *Agave* L. accessions containing different ploidy levels (*A. tequilana* Weber $2n=2\times=60$, *A. angustifolia* Haw. $2n=2\times=60$ and $2n=6\times=180$, and *A. fourcroydes* Lem. $2n=3\times=90$ and $2n=5\times=150$). Ploidy level analysis in these plants might help explain the evolutionary mechanisms implied by the high diversity (i.e., fast speciation) and genetic variability reduction found in some extensively cultivated species of this genus (i.e., *A. tequilana* Weber). In addition, upon identification of ribosomal DNA (rDNA) haplotypes, a more detailed investigation was performed in order to gain information on the evolutionary relationships among these economically important *Agave* L. species.

Material and Methods

Biological Material

Three commercial *Agave* L. species were selected *A. tequilana* Weber, 1902 'Azul' ($2n=2\times=60$); two varieties of *A. fourcroydes* Lemaire, 1864 classified as 'Kitam ki' ($2n=3\times=90$) and 'Sac ki' ($2n=5\times=150$); and two varieties of *A. angustifolia* Haworth, 1915 classified as 'Marginata' ($2n=2\times=60$) and 'Chelem' ($2n=6\times=180$). *A. tequilana* Weber 'Azul' has been propagated in vitro and adapted to greenhouse conditions with commercial aims. Ploidy was previously estimated by Robert et al. (2008), and the accessions form part of the botanical garden of the Research Center of Yucatan (CICY), México.

Ribosomal Gene Copy Number

Copy number was characterized in the 5S and 18S rDNA loci of all *Agave* L. accessions. This was achieved through normalization of the DNA content using a nucleic isolation strategy. Secondly, since it was previously reported that the 4C DNA content varies widely among different *Agave* L. species (Table 1) (Palomino et al. 2003; Greilhuber et al. 2005; Moreno-Salazar et al. 2007; Robert et al. 2008), the extraction of isolated nuclei was conducted according to the procedures given by Zhang et al. (1995). Nuclei integrity was confirmed by optical microscopy, and their quantification was carried out using a hemocytometer. Genomic DNA was obtained by following the silica method described by Echeverria-Machado et al. (2005). Quantity, quality, and purity of the DNA were evaluated using NANODROP equipment (NANODROP, Promega, USA). Ribosomal copy number was determined by using quantitative capillary electrophoresis with primers obtained from a consensus sequence derived from several plant ribosomal sequences, specifically the Liliopsida class and Asparagaceae family for 5S and 18S rDNA, respectively. These primers have recently been used to characterize a binary genomic library of *A. tequilana* Weber (Tamayo-Ordóñez et al. 2012). Primer sequences are as follows: 5S (FW 5′-CGATCATACCAGCACTAAAGCACC-3′ and RV 5′-ATGCAACACGAGGACTTCCCAG-3′) and 18S (FW 5′-CGGCTACCACATCCAAGGAA-3′ and RV 5′-GCTGGAATTACCGCGGCT-3′). The alignment and localization of these primers are reported in the "Results" section (Supplementary Figs. A-1 and A-2). These primers were labeled with WellRED dyes D2-PA and D3-PA (Beckman Coulter, USA). PCR amplification conditions were performed in 20 μL of PCR reaction mix containing 2 μL of Master Mix (2×), 5 units of Taq polymerase (Gibco-BRL, USA), 0.5 pmol of each primer, and 50 ng of template DNA. Amplification was done with an initial denaturation of 3 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 65 °C (5S rDNA) or 63 °C (18S rDNA), and 1 min at 72 °C with a final extension of 10 min at 72 °C. PCR products were checked in agarose gels at 1 %, purified by Wizard® SV Gel and PCR Clean-Up System (PROMEGA, USA), and then electrophoreted in CEQ™ 8800 sequencer (PerkinElmer Inc., USA). Electropherograms obtained were analyzed by using

Table 1 Informative summary of the analysis of ribosomal regions (5S and 18S) in the different *Agave* L. accessions

Agave L. accession	Ploidy level (*x*)	4C DNA content (pg)[a]	rDNA 5S			rDNA 18S			5S/18S rate
			Copy number per genome[b]	Copy number per haploid genome[b]	Peaks detected	Copy number per genome[b]	Copy number per haploid genome	Peaks detected	
A. tequilana Weber	2	15.23	3,656,893	1,828,447	4	2,044,281	1,022,141	1	1.78
A. angustifolia Haw.	2	n.d.	4,803,740	2,401,870	4	2,493,065	1,246,532	1	1.92
A. angustifolia Haw.	6	45.24	16,657,531	2,776,255	4	8,742,755	1,457,126	1	1.90
A. fourcroydes Lem.	3	22.56	7,021,103	2,340,368	4	3,461,762	1,153,921	1	2.02
A. fourcroydes Lem.	5	37.46	11,795,006	2,359,001	4	5,304,455	1,060,891	1	2.22

n.d. not detected

[a] As is reported by Robert et al. (2008)

[b] Calculated considering the sum of all peak areas detected in the capillary electrophoresis

the software GeneMarker v.1.75 (PerkinElmer, Inc., USA), which allow discrimination among different allelic sizes for each rDNA gene based on BioVentures size fragment standard 400 (BioVentures, Murfreesboro, USA). All peaks detected in the electropherograms were used to build the standard curves for each gene (5S and 18S rDNA). First, the DNA content was calculated in about 30,000 nuclei mL^{-1}, for each *Agave* L. accession, across several nuclei concentrations (10; 100; 1000; 10,000; and 100,000 nuclei mL^{-1}). The amount of nuclei was previously standardized in all specimens to 12,000 nuclei mL^{-1}. Second was carried out using a template of different amounts of plasmidic DNA contained in the corresponding ribosomal sequences (0.001, 0.01, 0.1, 1, 10, 100 ng μL^{-1}). The ribosomal copy number, in 12,000 nuclei mL^{-1}, was therefore calculated for each *Agave* L. accession analyzed. The number of rDNA copies per genome was determined in relation to the area under each peak (fluorescence intensity).

Haplotype Analysis of rDNA Genes

Capillary electrophoresis analysis on rDNA gene (5S and 18S) was also applied to determine different gene size peaks in all *Agave* L. accessions. PCR amplification and capillary electrophoresis conditions were similar to those reported in the previous section. 5S rDNA gene shows the presence of four peaks with similar length (bp). Therefore, a more detailed analysis of these rDNA genes was conducted. PCR ribosomal products were cloned in the pGEM-T vector (Promega, USA) using competent *E. coli* INVαDH10b cells according to the manufacturer's instructions. At least ten clones of rDNA gene for each *Agave* L. accession were chosen to sequence using universal M13 primers and sequenced using MACROGEN (Seoul, Korea) NGS sample submission (ABI 3730 XL). The nucleotide sequences were aligned and compared with those at the GenBank database using the BioEdit Sequence Alignment Editor 5.0.6 (Hall 1999) and CLUSTAL W 1.82 software (Thompson et al. 1994). The dendrograms were built by an analytical program (MEGA version 5.1, Tamura et al. 2011) using the minimum-evolution (ME) method and the Jukes-Cantor method for inferred evolutionary distances. The reliability of the clusters was evaluated by bootstrapping with 1000 replicates.

Ribosomal sequences were also evaluated by constructing a minimum haplotype network using the median-joining method implemented in Network v.4.6 software (http://www.fluxus-engineering.com; Bandelt et al. 1999), assuming an epsilon of 0 and a transversion/transition ratio of 1:2. Haplotype polymorphisms are showed in relation to the sequences FJ882491 (*Triticum turgidum*) and GU980213 (*Agave tequilana*) for 5S and 18S rDNA genes, respectively (Supplementary Figs. B-1 and B-2). Codes by species and ploidy events were as follows: *A. tequilana* $2n=2\times=60$: At2X; *A. fourcroydes* $2n=3\times=90$ and $2n=5\times=150$: Af3X, Af5X; and *A. angustifolia* $2n=2\times=60$ and $2n=6\times=180$: Aa2X, Aa6X, respectively.

Modeling of Secondary Structure

RNA fold prediction was carried out using the RNAstructure Web Server (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) (Reuter and Mathews 2010), at a folding temperature of 37 °C. The RNAz program of Vienna RNA web servers (http://rna.tbi.univie.ac.at/) (Gruber et al. 2007) was used to test the probability of functionality of predicted secondary structures. Sequences were analyzed in the reverse orientation as a control.

rDNA Expression

High-quality RNA was obtained from a constant number of nuclei regardless of the ploidy levels of the *Agave* L.

accessions assayed. We used three biological replicates per species analyzed. Samples were adjusted in all specimens to 30,000 nuclei mL^{-1}, and nuclei isolation was carried out as previously described. RNA isolation was conducted using the TRIzol method (Invitrogen, USA) according to the manufacturer's protocol. RNA integrity and quantification were verified 1.3 % agarose gel after electrophoresis and by spectrometry using the A260/A280 and A260/A230 ratios in NANODROP (NANODROP-1000, Thermo Scientific, USA). One microgram total RNA was digested with 1 U RQ1 RNase-Free DNase I (PROMEGA) and DNase 1× reaction buffer in a final volume of 10 μL. The samples were incubated at 37 °C for 30 min. Subsequently, the DNase I was inactivated by adding 1 μL of RQ1 DNase Stop Solution incubating at 65 °C for 10 min. The absence of DNA contamination was verified by agarose electrophoresis (1.3 %) and negative PCR, using as a template 50 ng of RNA treated with DNase I. In this PCR, a sample of genomic DNA was included as a positive control. The primers used were 5S rDNA gene forward 5′-CGATCATACCAGCACTAAAGCA CC-3′ and reverse 5′-ATGCAACACGAGGACTTCCCAG-3′. PCR amplification conditions were an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, annealing temperature 65 °C for 1 min, and 72 °C for 1 min with a final extension of 72 °C for 10 min.

The complementary DNA (cDNA) was synthesized using the GoScript™ Reverse Transcription System (PROMEGA, USA). To prepare the cDNA, 1 μg of RNA treated with DNase I was mixed with 0.5 μg $Oligo(dT)_{15}$ and Random Primer. This reaction was incubated to 70 °C for 5 min immediately chilled in ice for 5 min. Subsequently, RT Master Mix was added, containing GoScript™ 1× Reaction Buffer, 5 mM $MgCl_2$, 1 μL PCR Nucleotide Mix (0.5 mM each dNTP), 1 U Recombinant RNasin Ribonuclease Inhibitor, and 15 U/μg GoScript™ Reverse Transcriptase, in a final volume of 20 μL. The reaction was incubated at 25 °C for 5 min, after that 37 °C for 1 h, and finally, the reaction was inactivated at 70 °C for 15 min. A control positive (1.2 kb Kanamycin Positive Control RNA) provided in the kit was used to estimate the yield of synthesis of cDNA. The concentrations of cDNA were verified by NANODROP measurements. The cDNAs obtained were stored at −60 °C until use.

Amplification of the 5S and 18S rDNA genes was standardized in a real-time thermal cycler (Applied Biosystems). Fifty nanograms of cDNA adjusted to 1 μL was used as template in 20 μL of PCR reaction mix, containing 5 μL of PCR Master Mix (2×) with SYBR Green (Applied Biosystems), 2 units of Taq polymerase (Gibco-BRL, Rockville, MD), and 0.5 pmol of forward and reverse primers. PCR amplification conditions were the same as described above for the determination of ribosomal gene copy number. With the aim of discard that originated fluorescence is the result of non-specific PCR products, primer concatenation is performed for melt-curve analysis. Controls of the negatives for reference gene and target gene were always included in the experiments in order to eliminate DNA contamination. The SYBR green fluorescent signal was standardized to a passive reference dye (ROX) included in the SYBR Green PCR Master Mix (Applied Biosystems P/N 4309158).

Finally, the relative expression of each gene was determined by the ΔΔCq method between the ribosomal gene and the reference gene (Pfaffl 2001; Nicot et al. 2005; Maroufi et al. 2010). Actin was selected as reference gene since in previous RT-qPCR analysis, it has proven to be a reliable reference gene (Torres-Maldonado et al. 2001; Choquer et al. 2003; Hacquard et al. 2011). Actin primers for *Agave* L. genus were obtained from a consensus sequence derived from several related species as follows: FW 5′-GGAGAAGA GTTATGAGCTGCCTGAC-3′ and RV 5′-CATAGCATCA GCAATACCAGGGAA-3′ (Supplementary Fig. A-3). The transcript abundance ratio of target gene to reference gene was determined by the following equation: relative expression$=(E_{ref})^{Ctref/}(E_{target})^{Cttarget}$, where E_{ref} and E_{target} are the efficiencies of the primers for the reference and target genes, respectively, and Ct_{ref} and Ct_{target} are the mean Ct values of reference and target genes, respectively (Pfaffl 2001).

Expressed copies of different allelic groups were quantified by using quantitative capillary electrophoresis of 5S rDNA peaks. This technique allows us to quantify the copy number of 5S rDNA allelic groups that differ in length until a single nucleotide based on a standard curve of plasmidic DNA as mention below. The primers 5S labeled with WellRED dye D3-PA were used for amplification of 5S ribosomal regions. PCR amplification mixture was performed in 20 μL of PCR reaction mix containing 2 μL of Master Mix (2×), 5 units of Taq polymerase (Gibco-BRL, USA), 0.5 pmol of each primer, and 50 ng of template cDNA. Amplification was done with an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, at 65 °C for 1 min, and at 72 °C for 1 min with a final extension of 10 min at 72 °C. PCR products were purified by using Wizard® SV Gel and PCR Clean-Up System (PROMEGA, USA) and then electrophoreted in CEQ™ 8800 sequencer (PerkinElmer Inc., USA). Electropherograms obtained were analyzed by using the software GeneMarker v.1.75 (PerkinElmer, Inc., USA). The value of fluorescence intensity of each peak (105, 107, 110, and 111 bp), was interpolated in a curve of plasmidic DNA containing a representative 5S rDNA gene for the peak IV (111 bp), due to its high R^2 value (0.998) and abundant peak area. Plasmidic template amounts applied in the curve were 0.001, 0.01, 0.1, 1, 10, and 100 ng μL^{-1}. The values of intensity of fluorescence obtained in each dilution of plasmidic DNA were related with a number of copies of the gene 5S rDNA. The rationale was based in that 1 pg of plasmidic DNA is equivalent to 960 kb (Arumuganathan and Earle 1991), and genetic construction (vector of cloning and insert) has a size of 3111 bp, indicating

that there are 308,500 copies of gene 5S rDNA in 960 000 bp (corresponding to dilution 0.001 ng). For each dilution, the number of copies was estimated and related with an intensity of fluorescence. The interpolation of values of intensity of fluorescence obtained in the samples of cDNA with the standard curve allowed calculating the number copies expressed of each peak 5S rDNA for this method.

Results

Informative Genetic Parameters Analyzed Between *Agave* L. Accessions

Informative genetic parameters in these *Agave* L. accessions were characterized in two rDNA genes (5S and 18S) (Table 1). Considering the copy number per genome, independently of the ribosomal region, an increase in the ploidy level was observed. 18S rDNA copy number varies widely among the different ploidy levels of each accession, and this was highlighted in the variation observed in *A. angustifolia* Haw., which showed a difference of more than 6,249, 690 copies (3.5-fold) between the variety Chelem ki (hexaploid) and the variety Marginata (diploid). Similarly, in *A. fourcroydes* Lem., there was an increase of 1,842,693 copies (1.53-fold) in the variety Sac ki (pentaploid) in comparison to the variety Kitam ki (triploid). It is important to mention, however, that if this comparison is conducted only between diploid varieties, *A. angustifolia* Haw. exhibits 1.22 times more copies when compared to *A. tequilana* Weber. In fact, when this comparison is conducted with the lower ploidy level of *A. fourcroydes* Lem. 'Kitam ki', the difference was 1.69 times more copies in the triploid specimen. 5S rDNA copy number also shows variations following a similar tendency with regard to the 18S gene. However, the relations between different polyploidy accessions per species were 3.47 times more for the *A. angustifolia* Haw. hexaploid in relation to the diploid and 1.67 times more for *A. fourcroydes* Lem. pentaploid in relation to the triploid variety. 5S rDNA copy number was also reduced in *A. tequilana* Weber since *A. angustifolia* Haw. (diploid) and *A. fourcroydes* Lem. (triploid) exhibit 1.31 and 1.92 more copies, respectively, that the diploid variety of this species. In fact, *A. tequilana* Weber always showed the lowest copy number of both ribosomal genes. In general, these results showed a wide range of variation in rDNA gene proportion in a species-specific way, but the 5S/18S copy number rates are similar with two times more copies of the 5S rDNA gene (Table 1). Specific expression for different 5S rDNA alleles was recorded and will be discussed.

DNA Haplotype Identification

An increase in rDNA copy number is related with the ploidy level in the different *Agave* L. accessions. A more detailed analysis of these ribosomal genes was conducted. For this, PCR products relative to different rDNA genes (5S and 18S) were evaluated by capillary electrophoresis. This method allows a fast discrimination among different peak sizes relative to these marker genes. Special care was taken in the design of the universal primers used for this amplification since the primers must be able to amplify different haplotypes occurring in these different *Agave* L. accessions. For this, the 5S rDNA primers were designed by aligning different sequences obtained from the class Liliopsida. This class includes the Asparagaceae family, which only have a 5S rDNA sequence in the GenBank database. The region sequence predicted by using these primers is highly conserved in the class with a range of about 111 bp. Similarly, 18S rDNA primers were also designed by aligning different sequences obtained from the Asparagaceae family. This family includes the genus *Agave* L., which only has three 18S rDNA sequences in the GenBank database. The region sequence predicted by using these primers is highly conserved in the family with a range of about 187 bp (Supplementary Figs. A-1 and A-2). One minor peak also was detected, but this was out of predictable range with a fluorescence intensity of scarcely 2 to 3 % of that detected in the major peak (187 bp). Therefore, this minor peak was considered as an unspecific PCR amplification, and consequently, it was discarded from the analysis. Results obtained for the 5S rDNA PCR amplification show an electropherogram profile including four peaks of about 105, 107, 110, and 111 bp with the later more abundant. This profile was consistent in all *Agave* L. accessions analyzed (Fig. 1). 18S rDNA amplification was also conserved in all *Agave* L. accessions showing only one peak at about 187 bp (Supplementary Fig. C). This information is summarized in Table 1.

The presence of different peak sizes for each rDNA amplification product, at least for 5S rDNA, in the different *Agave* L. accessions suggested the presence of at least four haplotypes, with different sequence length, for this rDNA gene. Therefore, a sequencing analysis was incorporated in this study and at least ten different clones were selected for 5S rDNA in all *Agave* L. accessions to sequence giving 55 sequences in total. Preliminary phylogenetic analysis of these clones with regard to members of the class Liliopsida and the family Asparagaceae for 5S and 18S rDNA clones, respectively, was analyzed by using minimum-evolution trees (Fig. 2). No clear relationships among different clones were obtained, although all *Agave* L. clones for 5S rDNA were separately distributed with regard to other Liliopsida species, whereas clones for 18S rDNA were more closely related to the *Agave*

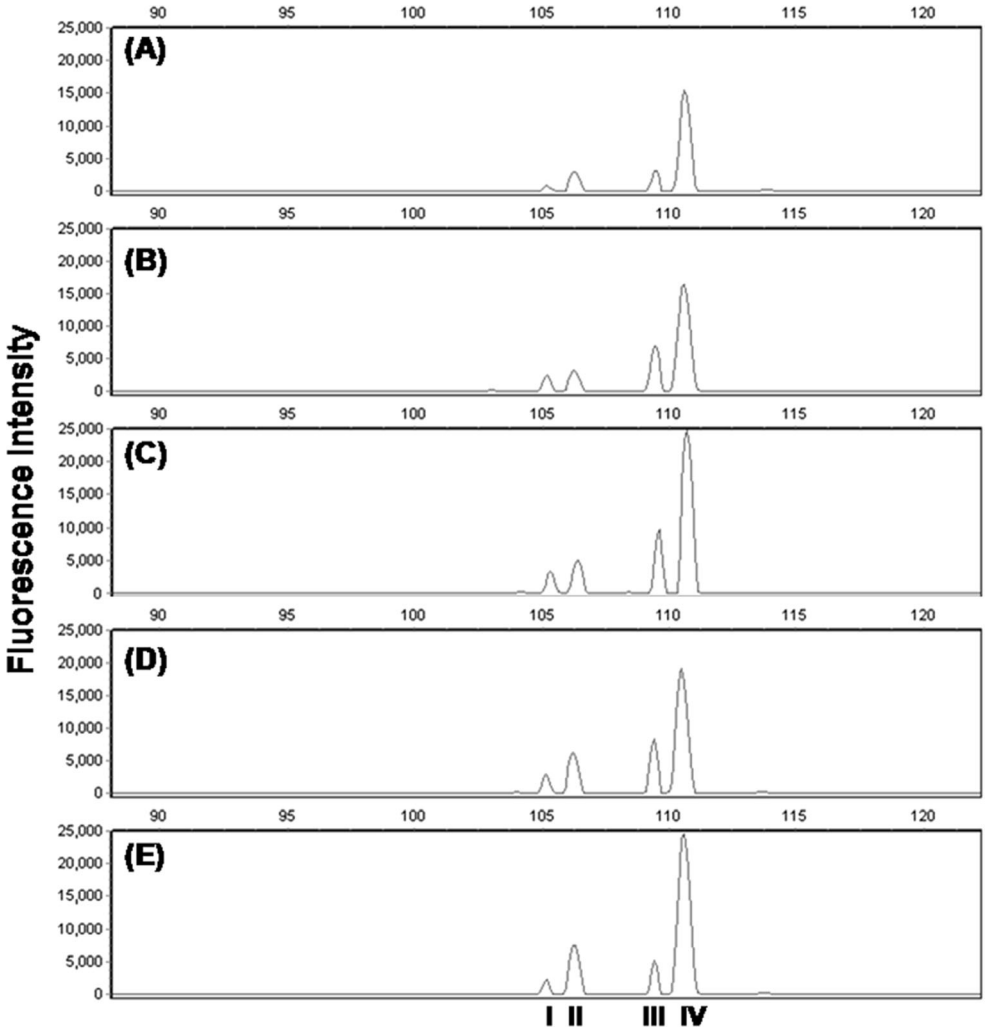

Fig. 1 Examples of electropherograms obtained from different *Agave* L. accessions using 5S rDNA PCR products. **a** *A. tequilana* Weber ($2n=2\times=60$), **b** *A. angustifolia* Ham. ($2n=2\times=60$), **c** *A. angustifolia* Ham. ($2n=6\times=180$), **d** *A. fourcroydes* Lem. ($2n=3\times=90$), and **e** *A. fourcroydes* Lem. ($2n=5\times=150$). Peaks numbered as *I*, *II*, *III*, and *IV* were about 105, 107, 110, and 111 bp, respectively

reference members (i.e., *A. tequilana* Weber 'Azul' and *Agave ghiesbreghtii* Lem.) and separate from the other Asparagaceae species (Fig. 2).

Following from the 5S rDNA distance tree, it was not possible to detect the presence of four allelic groups relative to the electrophoresis peaks (Fig. 1). However, it was possible to detect some clone clades as formed by 10At2X, 11At2X, 1Aa6X, 2Aa6X, 3Aa6X, 4Af3X, 1Af5X, and 3Af5X, which have different *Agave* species and different ploidy events. Therefore, the possibility of more than four shared haplotypes among these different *Agaves* L. accessions should be considered. This is also supported by the sequencing analysis of 37 clones in 18S rDNA (Fig. 2) where a clearly separate clade occurs, including the clones 6Aa2X, 10At2X, and 7Aa2X. Yet despite this, capillary electrophoresis shows only one peak (Supplementary Fig. C).

A more detailed analysis of the haplotypes was conducted to establish the minimal haplotype network in these *Agave* L. accessions, as shown in Fig. 3. Here, it is clear that there are at least three haplotype groups, which correspond to the capillary electrophoretic peaks II, III, and IV with clone sequences of 107, 110, and 111, respectively. No members of the peak I of 105 bp were cloned in any *Agave* L. accession, probably as consequence of its lower abundance (see Fig. 1 and Supplementary Fig. D). A total of 17 different haplotypes were determined using this strategy and were named HAP-01 to HAP-17. Interestingly, haplotypes with different sizes according the preliminary in silico analysis of 111 bp (Supplementary Fig. A-1), such as 110 (group III) or 107 bp (group II) haplotype groups, seem to have evolved separately since they exhibit a specific number of polymorphisms (i.e., HAP-2 of group III has polymorphic nucleotide changes in the aligning positions 31, 44, 41, 27, 81, and 82) (Fig. 3). Similarly, the minimal haplotype network for 18S rDNA gene was analyzed and only exhibited one capillary electrophoresis peak of about 187 bp (Supplementary Fig. C). Fourteen different haplotypes were determined, and most of them (64.2 %) showed 187 bp as expected; those shown to have different sizes exhibited 185 bp (HAP-03, HAP-08, and HAP-14), 184 bp (HAP-13) and 174 bp (HAP-10). This last haplotype

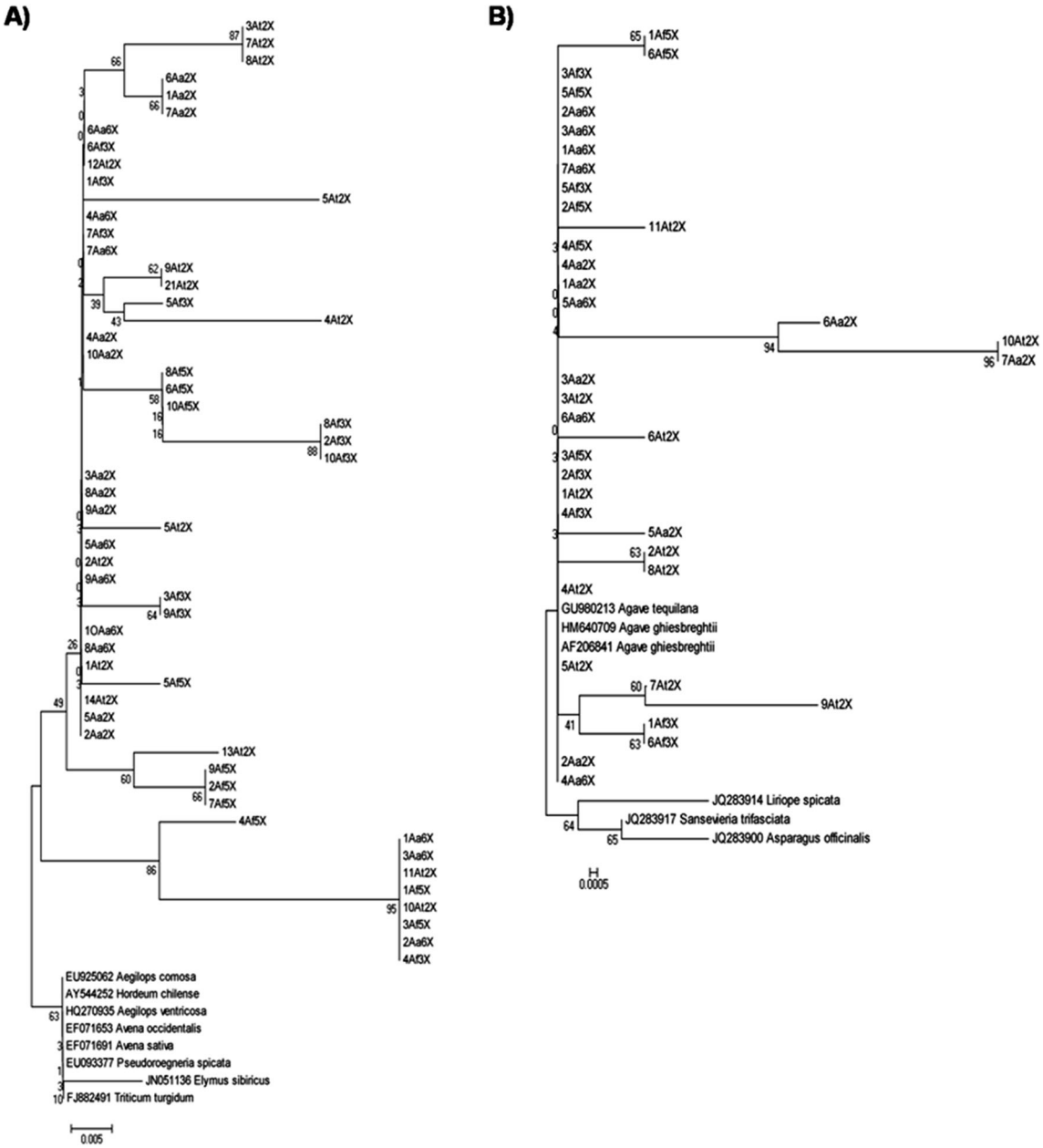

Fig. 2 **a** 5S rDNA distance tree. Reference species belong to the class Liliopsida, which includes the family Asparagaceae. **b** 18S rDNA distance tree. Reference species belong to the family Asparagaceae which includes the genus *Agave* L. Sequence codes are as follows: *Numbers at the beginning* are clone numbers; then, *At*, *Aa*, and *Af* are *A. tequilana* Weber, *A. angustifolia* Ham., and *A. fourcroydes* Lem., respectively. $2n=2\times=60$, $2n=3\times=90$, $2n=5\times=150$, and $2n=6\times=180$ are ploidy levels. Reference species were retrieved from GenBank. Each species accession number is shown at the beginning of its name

also had 15 polymorphic nucleotides, suggesting a major evolutionary distance. Regardless of the occurrence of different haplotype sizes in this ribosomal gene marker, only one specific capillary electrophoretic peak (187 bp) was detected, which suggests a low abundance of these haplotypes. Haplotypes with different sizes up to 187 bp only correspond to accessions with ploidy level of $2n=2\times=60$ (*A. tequilana* Weber 'Azul' and *A. angustifolia* Haw. 'Marginata').

For both rDNA genes (5S and 18S), those haplotypes with a major polymorphic changes and sizes (bp) also exhibited higher genetic distances in the phylogenetic trees. For example, the HAP-02 of 110 bp containing the clones 10At2X, 11At2X, 1Aa6X, 2Aa6X, 3Aa6X, 4Af3X, 1Af5X, and 3Af5X is very distant in the 5S rDNA tree, and the HAP-10 of 174 bp containing the 11At2X clone is also very distant in the 18S rDNA tree. From the analyses shown in Figs. 3 and 4, it is also possible to show that the more abundant haplotypes also correspond to the more abundant electrophoretic peaks (i.e., peak IV in Fig. 1 and see Supplementary Fig. D) and its size corresponds to that expected in the previous in silico

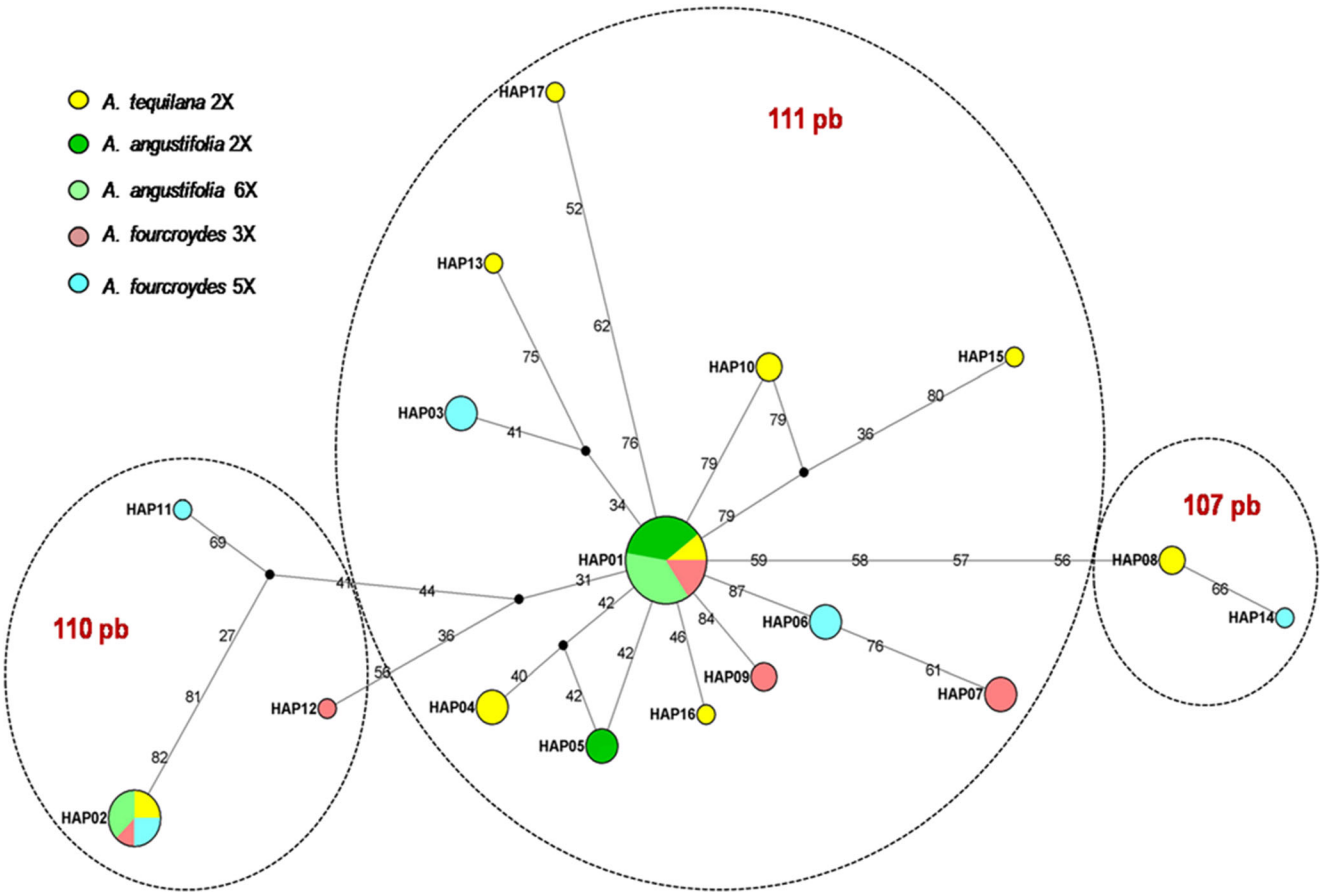

Fig. 3 Minimum haplotype network based on 5S rDNA from different *Agave* L. accessions. Haplotype color representing different *Agave* L. accessions is shown in the *right upper border*. Missing haplotypes are indicated as *small red circles*. The *line* connecting haplotypes represents one mutational step, whereas *numbers along the lines* are the specific nucleotide positions where the mutations occurred. *Circle area* is proportional to the frequency of each haplotype. *Doted circles* showed haplotypes of the same size (bp) range

analysis using these universal primers (Supplementary Figs. A-1 and A-2). These haplotypes (HAP-01 in both rDNA genes) were shared and redundant among all *Agave* L. accessions (Figs. 3 and 4). Specifically, in the 5S rDNA gene, the second more abundant haplotype (HAP-02) corresponding to the electrophoretic peak III (Group III) was redundant (Figs. 1 and 3). In this gene, only HAP-01 and HAP-02 were redundant. Interestingly, all *Agave* L. accessions exhibit non-redundant haplotypes in both rDNA genes (circles of the same color in Figs. 3 and 4) with the exception of *A. angustifolia* Haw. (hexaploid), which do not show any non-redundant haplotypes. On the contrary, *A. tequilana* Weber (diploid) has the highest number of non-redundant haplotypes, including seven haplotypes for each rDNA gene (Figs. 3 and 4). This accession also showed the lowest copy number of both ribosomal loci (Table 1).

The presence of non-redundant haplotypes for these marker genes which can be found in most *Agave* L. accessions (mainly in *A. tequilana* Weber) suggests a possible evolutionary strategy for this generation of asexually propagated *Agaves* L. accessions (Colunga-GarcíaMarín and May-Pat 1997; García-Mendoza 2007). Ribosomal genes are widely conserved (Long and David 1980; Barciszewska et al. 2000; Eickbush and Eickbush 2007); however, the generation of new specific haplotypes, as consequence of a faster evolutionary process, might originate either as functional or non-functional new haplotypes. To compare the predictable functionality of these non-redundant ribosomal haplotypes with regard to the most abundant and redundant haplotypes (HAP-01), a structural bioinformatics-assisted analysis was conducted. Minimal free energy structures were selected for 5S rDNA HAP-01, HAP-02, and HAP-08 since these are representatives of allelic groups II, III, and IV (Fig. 5). In addition, the first two haplotypes (HAP-01 and HAP-02) are shared among most *Agave* L. accessions and the last one (HAP-08) is exclusive for *A. tequilana* Weber. It was not possible to select any redundant haplotype in this group II (107 bp) since it was not identified. Finally, all selected sequences were analyzed with 5S rDNA sequences of the class Liliopsida (*Elymus sibiricus* L., *Hordeum chilense* Roem. et Schultz, and *Avena occidentalis* L.) retrieved from GenBank with the aim to compare their predicted secondary structures.

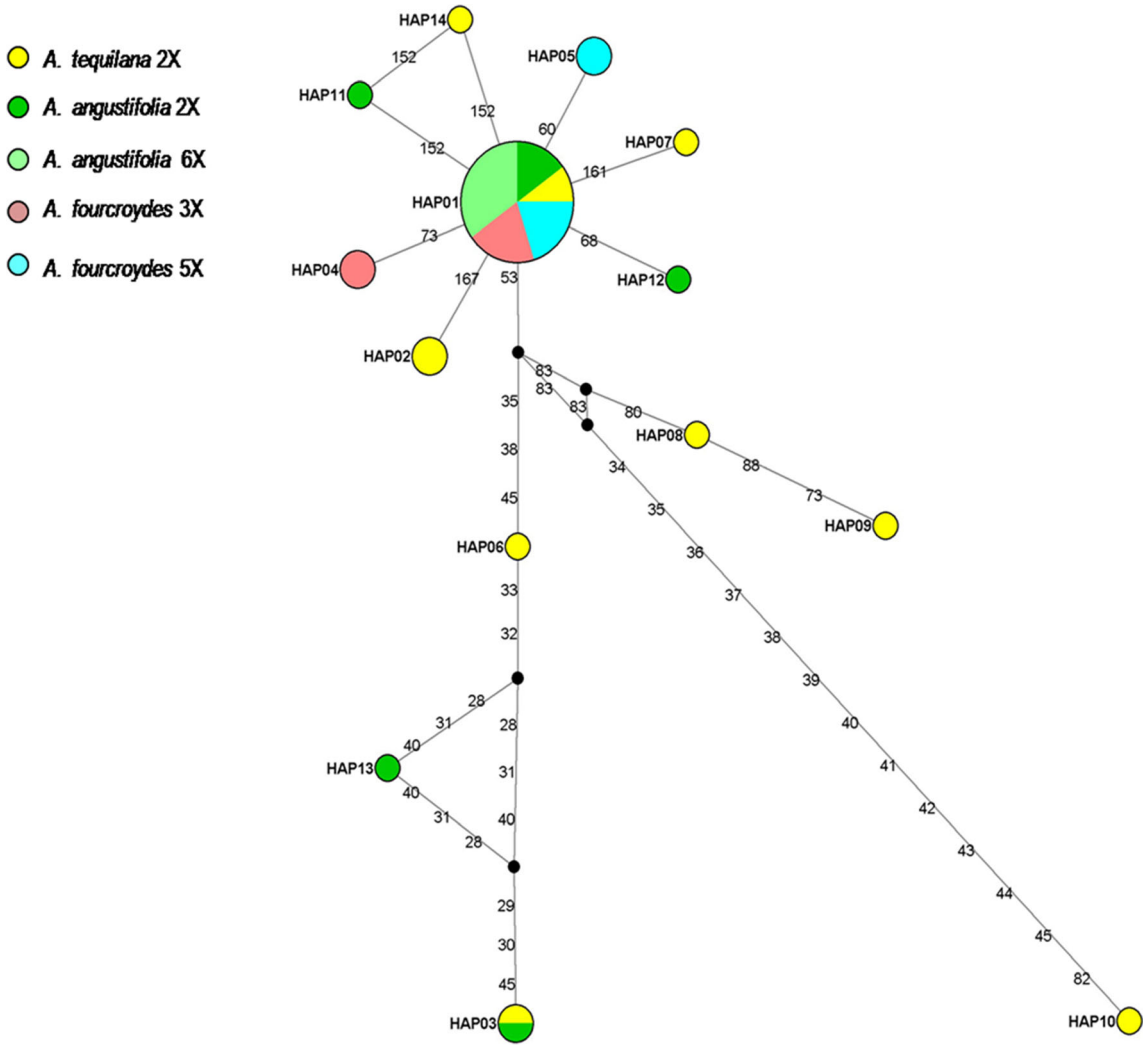

Fig. 4 Minimum haplotype network based on 18S rDNA from different *Agave* L. accessions. Haplotype color representing different *Agave* L. accessions is showed in the *right upper border*. Missing haplotypes are indicated as *small red circles*. The *line* connecting haplotypes represents one mutational step, whereas *numbers along the lines* are the specific nucleotide positions where the mutations occurred. *Circle area* is proportional to the frequency of each haplotype

All closest Liliopsida class sequences used showed secondary structures that fold into the structure derived from X-ray crystallography (Szymanski et al. 2002; Szymański et al. 2003). Only HAP-01 exhibits the conformation with its minimal free energy (ΔG=−40.1) being similar to others obtained by using the 5S rDNA sequences retrieved from GenBank (Fig. 5). Differently, HAP-02 (redundant; group III) and HAP-08 (non-redundant; group II) exhibit multiple secondary structures with ΔG values ranged of −32 to −29.2, and −36 to −34.7, respectively. Figure 5e, f only shows the conformations closest to X-ray crystallography model for these haplotypes. Occurrences of these multiple predictable secondary conformations regardless of their redundancy were used to analyze other haplotypes belonging to abundant allelic group IV and with multiple predictable secondary conformations. For this, the HAP-17 was selected due to having multiple secondary conformations and its non-redundant nature. A secondary structure of this haplotype is also slightly similar to X-ray crystallography model with ΔG value of −33.2 (Fig. 5f). In silico functionality of these haplotypes with multiple predictable secondary conformations was also explored by a structure functionality test, as mentioned above. Results showed that the redundant haplotypes within the allelic groups IV (HAP-01) and III (HAP-02) were functional with a *z*-score of −2.44 and −0.80, respectively. Differently, non-redundant HAP-08 and HAP-17 from allelic groups II and IV were not functional with *z*-score of −1.63 and −0.76, respectively (Supplementary Table A).

Comparative Characterization of the Relative rDNA Gene Expression Among Different *Agave* L. Accessions

Functionality differences found in 5S rDNA haplotypes in the different *Agave* L. accessions were conducted to analyze their

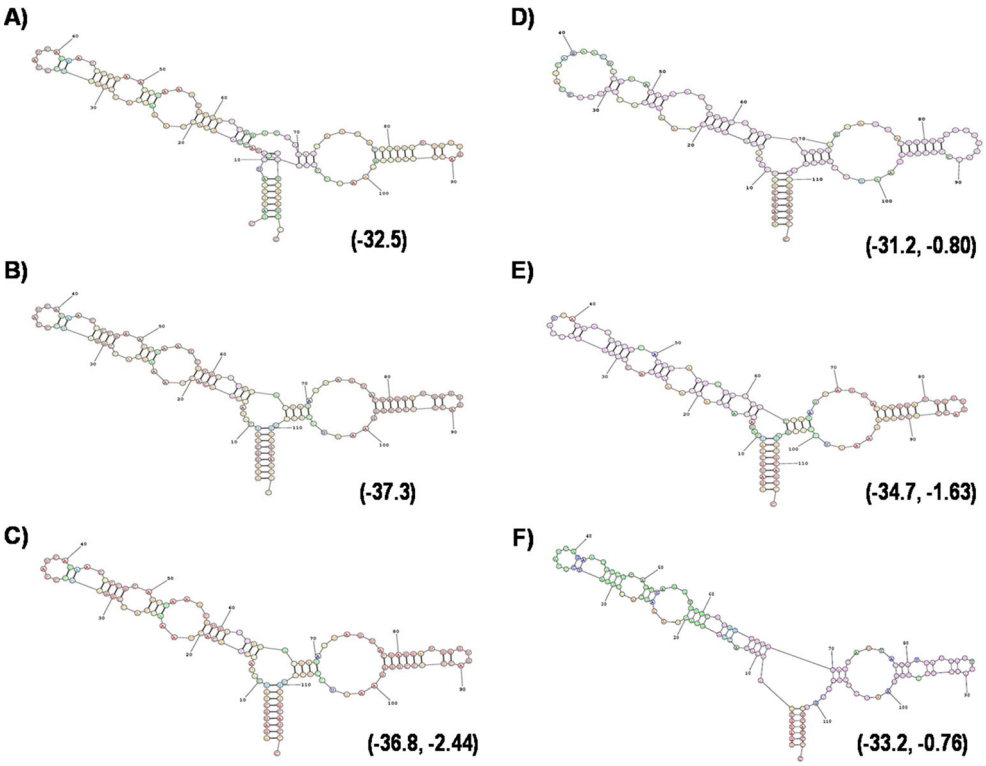

Fig. 5 Secondary 5S rDNA structure of *Agave* L. haplotypes and other species related of the class Liliopsida. *Agave* L. haplotypes (HAP) were complemented with the consensus 5′ and 3′ terminal sequences, GGAUG and CCC, respectively. Related 5S rDNA *Agave* L. genus and HAP-01 figures showed the lowest free energy conformation. HAP-02, HAP-08, and HAP-17 showed the conformations closest to X-ray crystallography structure. **a** *Elymus sibiricus* L. (JN051136), **b** *Hordeum chilense* Roem. et Schultz. (AY544252), **c** redundant HAP-01 (allelic group 4), **d** HAP-02 (allelic group 3), **e** non-redundant HAP-08 (allelic group 2), and **f** non-redundant HAP-17 (allelic group 4). *Values on the right lower border* of each plot shows free energy (ΔG) and functional test (z-score) values, respectively

expression in more detail. Firstly, a total expression analysis was done by real-time PCR. A normalized curve to estimate the rDNA relative expression was used with the actin gene and a constant cell number. Real-time PCR for 5S gene shows a constant expression in contrast with 18S, which exhibits a more variable expression in *A. angustifolia* Haw. (diploid) (Fig. 6a). Only *A. tequilana* Weber (diploid) exhibits a constant expression of both rDNA genes, suggesting a major regulation of these different rDNA transcripts.

In addition, expression analysis was also conducted on the 5S rDNA peaks detected in capillary electrophoresis in relation to the rDNA copy number (Fig. 6b). Each electrophoretic peak was considered as a different allelic group since previous analysis by minimal haplotype network suggested that these groups evolved separately (Fig. 3). Gene expression behaviors vary widely among the different *Agave* L. accessions, and no relation among the copy number of each allelic group and its expression level (i.e., allelic group IV in hexaploid *A. angustifolia* Haw. and pentaploid *A. fourcroydes* Lem.) was observed. However, when considering each *Agave* L. accession separately, the more abundant peaks III and IV also exhibited the greater transcript amounts, with exception of *A. tequilana* Weber where peak III was more expressed (Supplementary Table B). Finally, *A. tequilana* Weber (diploid) and *A. fourcroydes* Lem. (triploid) have no transcripts belonging to allelic groups I and II (Fig. 6b).

Discussion

Usefulness of 5S and 18S rDNA Genes in Ploidy and Evolutionary Studies in the *Agave* L. Genus

Ploidy analysis was conducted by using ribosomal genes since these are highly redundant and transcriptionally regulated (Flavell et al. 1986; Preuss and Pikaard 2007; Kovarik et al. 2008). In *Agave* plants, these genes have two loci (5S and 45S) present on different chromosomes and there is an apparent constant increase in loci according to ploidy level (Robert et al. 2008). In general, rDNA genes are members of a multigene family with the ability to maintain their sequence homogeneity. This ability to change their sequence in a highly synchronized manner has been described as concerted evolution (Arnheim et al. 1980; Dover 1982; Drouin and Moniz de Sá 1995). For an rDNA analysis in *Agave* plants, it was first necessary to overcome the technical difficulty of rDNA amplification. In these plants, 5S rDNA *Agave* L. gene sequences were not previously reported in GenBank. Similarly, for 18S rDNA, there are only a few *Agave* L. sequence accessions. Therefore, in this study, ribosomal primers are proposed for ploidy studies and designed to encourage the amplification of not only *Agave* L. accessions but also other plant members, exploring their possible use in phylogenetic analyses. The design of these primers thus includes members of the Liliopsida class and Asparagaceae family for 5S and 18S rDNAs, respectively (Supplementary Fig. A). These ribosomal sequences have been widely used in phylogenetic studies of

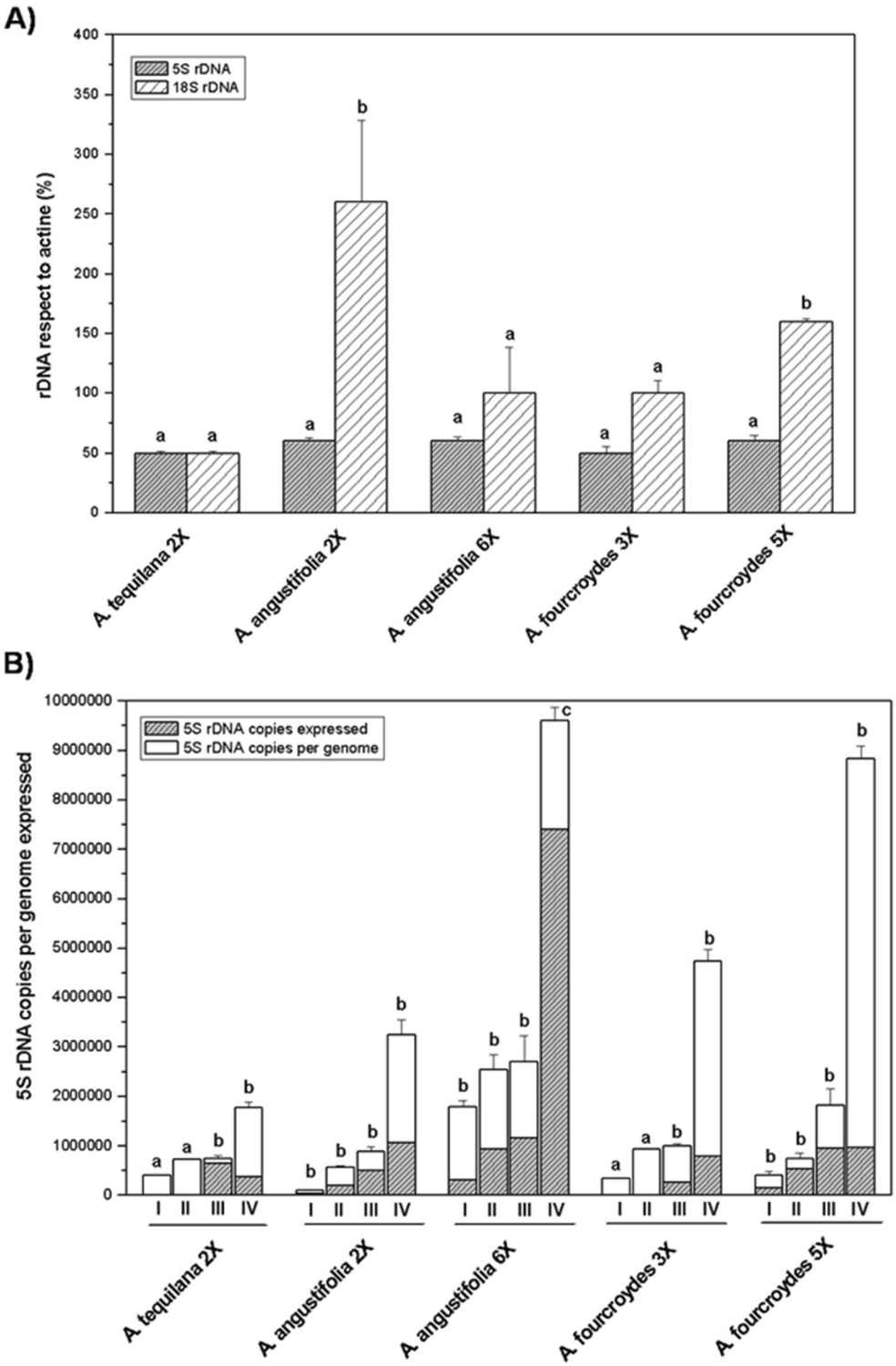

Fig. 6 Expression analysis of rDNA genes. Expression analysis was obtained from 12,000 nuclei mL^{-1} for each *Agave* L. accession. **a** Total rDNA expression was obtained by real-time PCR using specific primers. Total expression was normalized by using the actin gene. *Black bars*: 5S rDNA gene. *White bars*: 18S rDNA gene. **b** Specific 5S rDNA expression for each detected peak in capillary analysis (*number in top of each stacked bar* according to Fig. 1). Specific expression is presented as a percentage of 5S rDNA total copy number (*white region in stacked bar*). *Error bars* represent the standard error (n=3). *Different letters in the bars* represent the statistical significance of mean differences between each determination at an allelic group according to the Tukey test ($P \leq 0.05$)

plants (Baldwin et al. 1995; Nishiyama and Kato 1999; Campo et al. 2009). Although better results have been obtained using non-transcribed 45S or 5S spacers in diploid non-hybrid organisms (Hsiao et al. 1995; Becerra 2003; Ng'uni et al. 2010), in this study, the trees built using 5S and 18S rDNA genes barely separated the *Agave* L. accessions from other closely related genera. The *Agave* genus comprises a high number of polyploidy species, probably allopolyploids, which potentially might complicate the rDNA-based phylogenetic analysis since this has been reported to show a high presence of divergent paralogues or pseudogenes (Buckler et al. 1997; Mayol and Rosselló 2001; Márquez et al. 2003;

Bailey et al. 2003). In addition, the rDNA genes transcribed, particularly 5S rDNA, seem to be less responsive to this evolution process and consequently are less useful to phylogenetic analysis (Rich et al. 1997; Fulnecek et al. 2002). In this study, rDNA tree analysis showed major genetic distances among some *Agave* L. accessions (>0.01), regardless of its ploidy level or species. This was also the case with other non-*Agave* plants (Fig. 2). Therefore, the results obtained here suggest that these gene loci are not useful to characterize phylogenetically different species in the *Agave* L. genus but might be more informative in evolutionary and ploidy studies. These 5S and 18S rDNA primers were also used to determine the copy number of these loci in the different *Agave* L. accessions. Results obtained show that both genes increase in copy number according to ploidy level, thus confirming the results previously obtained by Robert et al. (2008) (Table 1). The 5S/18S rDNA copy rates show a similar profile among different accessions with approximately double the number of copies for the 5S rDNA locus. A similar result has been obtained in other crops as *Oryza* L., *Tragopogon* L., *Paspalum* L., and *Centaurea* L. (Shishido et al. 2000; Pires et al. 2004; Vaio et al. 2005; Dydak et al. 2009).

Occurrence and Significance of Non-Redundant rDNA Haplotypes in *Agave* Plants

Differences found among some rDNA haplotypes, particularly for the 5S rDNA tree, conducted to carry out a more detailed analysis, identified 14 and 17 different rDNA haplotypes (redundant and non-redundant) for 5S and 18S loci, respectively (Figs. 3 and 4). *A. tequilana* Weber, in particular, shows a high amount of non-redundant haplotypes with seven for each rDNA gene. The presence of this genetic dynamism could be considered as a strategy of fast evolution due to the intensive cultivation and vegetative propagation of some of these species (e.g., *A. tequilana* Weber) (Colunga-GarcíaMarín et al. 1999; Gil-Vega et al. 2001; Infante et al. 2003; Gil-Vega et al. 2006). *A. tequilana* Weber has been under selection stress for several decades in comparison to *A. fourcroydes* Lem. and *A. angustifolia* Haw. (Vargas-Ponce et al. 2007; Gil-Vega et al. 2001), where the later species has the lowest management strategy and more contrasting ploidy levels in this study ($2n=2\times=60$ and $2n=6\times=180$). *A. angustifolia* Haw. (hexaploid) variety is also the only species that does not exhibit any non-redundant rDNA haplotypes in both rDNA genes. On the other hand, the occurrence of many non-redundant haplotypes in *A. tequilana* Weber (diploid), the species which is more intensively cultivated, might imply a reduction in its sequence homogenization. Gene homogenization depends on intra- or inter-chromosomal genetic exchange, copy number, and locus location (Dover 1982; Hillis and Dixon 1991; Suh et al. 1993; Wendel et al. 1995). In this study, two rDNA genes located on different chromosomes were analyzed with similar variability in the results. Also, copy number rates were also similar among different *Agave* L. accessions. Therefore, the changes in gene homogenization (non-redundant haplotypes) exhibited among the different *Agave* L. accessions could be due to an intra-chromosomal genetic exchange. These may be originating by locus rearrangements and rDNA tandem reorganizations (Levy and Feldman 2004; Pires et al. 2004; Pontes et al. 2004; Udall and Wendel 2006). This phenomenon, particularly observed in the 5S rDNA gene, is different to the concerted evolution model and may result in new gene variants by gene duplication. Some of these gene variants might diverge functionally and be maintained in the genome, while others become pseudogenes (Mayol and Rosselló 2001; Márquez et al. 2003; Bailey et al. 2003). Therefore, a functional analysis on these different redundant and non-redundant haplotypes was further conducted.

In Silico Analysis for Functional rDNA Haplotype

Functional divergence of redundant and non-redundant rDNA haplotypes found in the different *Agave* L. accessions was explored by predictable structural and transcriptional analysis. The maintaining of new haplotypes, some of which were non-functional, implies a specific transcriptional regulation (Jefrey and Pikaard 1997; Sandmeier et al. 2002; French et al. 2003). In this study, some redundant and non-redundant 5S rDNA haplotypes were observed; however, rDNA copy number only increased according to accession ploidy number. Similarly, in silico functionality analysis showed that redundant haplotypes HAP-01 and HAP-02 are functional whereas non-redundant HAP-08 and HAP-17 were non-functional. In general, all haplotypes detected exhibit secondary structures folded into the structure derived from X-ray crystallography (Szymanski et al. 2002; Szymański et al. 2003) and functionality seems to be more related with redundancy instead of allelic group (i.e., HAP-01 and HAP-17 belonged to the more abundant group IV). In this sense, it has been described that 5S rDNA genes seem to be less responsive to this concerted evolution process generating different polymorphic haplotypes (Fulnecek et al. 2002; Garcia et al. 2010), although some of these might not be functional or effectively transcribed (Garcia et al. 2012).

Total rDNA expression analysis showed a similar 5S rDNA transcript accumulation among all *Agave* L. accessions, regardless of their ploidy level (Fig. 6a). Total 18S rDNA transcripts exhibit some changes, but these were more related to the species than the ploidy level. Interestingly, the species more highly cultivated and with major non-redundant haplotypes, *A. tequilana* Weber, exhibits a similar total expression profile for both rDNA genes, implying a strong regulation of 5S rDNA under the 18S rDNA. This is similar to previous reports for several important crops such as tobacco, wheat,

and rose (Flavell et al. 1986; Dadejová et al. 2007; Kovarik et al. 2008; Khaitová et al. 2010). Two important observations help us to support a more detailed rDNA expression analysis in the more regulated 5S rDNA gene. Firstly, the presence of a multiple and consistent 5S rDNA capillary electrophoretic peak profile was observed. Secondly, the size (bp) of these peaks corresponded with the groups obtained by the minimal haplotype network analysis suggesting that these share a similar evolutionary history and might be considered as allelic groups (Figs. 1 and 3). Similar evolutionary rates in each allelic group may also be supported by specific transversion/transition rates (*R*) of each allelic group being 5, 6, and 4 %, for groups II, III, and IV, respectively (data not shown). Specific polymorphism occurrence and similar *R* values are characteristics shared among specific allelic groups of other important crop plants such as *Quercus*, *Eucalypt* L. Hér., *Lima bean* L., and *Artemisia* L. (Muir et al. 2001; Hartmann et al. 2001; Garcia et al. 2010; Serrano-Serrano et al. 2012). Therefore, and even when there are multiple 5S rDNA haplotypes, a specific transcriptional analysis was conducted by using these allelic groups (derived of capillary electrophoresis peaks profile). In general, 5S rDNA allelic groups I (105 bp) and II (107 bp) show the minor transcript accumulation. In fact, transcripts for these groups were entirely absent in *A. tequilana* Weber (diploid) and *A. fourcroydes* Lem. (triploid). Special attention should be given to the allelic group III in *A. tequilana* Weber since it exhibits the major transcription percentage (87 %) with regard to its rDNA copies. Transcriptional rDNA gene activation might be related with hybrid genetic vigor or allopolyploid species (Marmagne et al. 2010) and with additive expression of different rDNA copies (Bottley et al. 2006; Kovarik et al. 2008; Gaeta et al. 2009). Taking into account that *A. tequilana* Weber has the accessions with more non-redundant 5S rDNA haplotypes and that it exhibits a different expression profile with regard to other *Agave* accessions, it is possible to consider a specific evolutionary behavior for this *Agave* L. accession. These results seem to suggest a higher selection pressure, at least for allelic group III in *A. tequilana* Weber, probably as a consequence of the agronomic selection. However, it is important to mention that to support this argument, other related studies should be applied with other genes directly implied in traits involved with the agronomic strategy selection on this *Agave* accession.

In conclusion, the current study is the first report on commercial *Agave* L. accessions where the variability, functionality, and regulation of rDNA regions in terms of their ploidy level were explored. Total rDNA copy number was the main factor that corresponded with the ploidy level. However, the occurrence of different rDNA haplotypes was detected in all *Agave* L. accessions. Specifically, 5S rDNA haplotypes fall in different allelic groups that include both redundant and non-redundant haplotypes. These allelic groups also exhibit different regulation tendency. The agronomically important *A. tequilana* Weber species (diploid) was particularly different in terms of its non-redundant haplotypes and 5S rDNA transcription, suggesting an evolutionary differential pressure on this *Agave* L. accession.

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