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Frequencies and variation in cytosine methylation patterns in diploid and tetraploid cytotypes of *Paspalum notatum*

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Abstract

Paspalum notatum Flügge is a grass species organized as an agamic complex. The objective of the current research was to survey the frequencies and variation of cytosine methylation at CCGG sequences in diploid and tetraploid genotypes, and to determine the occurrence of methylation changes associated with tetraploidization by using methylation-sensitive amplification polymorphism (MSAP) markers. No differences were found in the average proportions of methylated CCGG sites between cytotypes, but methylation patterns were significantly more variable in tetraploids. In both groups of plants, epigenetic and non-epigenetic variation correlated significantly when compared by Mantel tests. The evaluation of 159 common MSAP markers showed that 18.86 % of them differed in their methylation status in the different ploidies. Dendrogram analysis, reflecting epigenetic distances, showed that the four diploids and one experimentally-obtained sexually-reproducing tetraploid, grouped together. MSAP analysis performed on a diploid plant and its autotetraploid derivative showed that new epialleles emerged after tetraploidization. Sequencing of several MASP markers showed homologies with low copy genes, non-coding sequences and transposon/retrotransposon elements.

Additional key words: agamic complex, apomixis, cytosine methylation, MSAP markers.

Introduction

The addition of a methyl group to the C5 position of a cytosine residue is the most common DNA modification in plants and animals (Cervera *et al.* 2002). DNA methylation is essential for viability and is involved in numerous biological processes, including embryogenesis and development, genomic imprinting, silencing of transposable elements, regulation of gene transcription and responses to stress (Martienssen and Colot 2001, Zilberman *et al.* 2007, Zhong *et al*. 2010). Epigenetic alterations appear to be especially prevalent during the formation of interspecific hybrids and polyploids (Liu *et al.* 2001, Shaked *et al.* 2001, Wang *et al.* 2004). Although more modifications take place after allopolyploidy, variations in cytosine methylation patterns, which correlated with the ploidy level, have been detected after autopolyploidization in *Eragrostis curvula* (Ochogavía *et al.* 2009). Methylation-sensitive amplification polymorphisms (MSAP) (Xiong *et al.* 1999) enable the evaluation of the levels and patterns of DNA methylation of a genome from a wide perspective. The methodology is based on the amplified fragment length polymorphism (AFLP) technique (Vos *et al.* 1995), but replacing the frequent cutting enzyme *Mse*I by the isoschizomers *Hpa*II and *Msp*I. Both enzymes recognize the same target sequence (CCGG) but exhibit different sensitivity to the methylation status (McClelland *et al.*

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Abbreviations: AFLP - amplified fragment length polymorphism; MIP - methylation-insensitive polymorphism; MSAP - methylation-sensitive amplification polymorphism; MsP - methylation-sensitive polymorphism; PCR - polymerase chain reaction.
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1994, Tardy-Planechaud *et al.* 1997).

Paspalum notatum Flüggé (bahiagrass) is a perennial rhizomatous grass species organized as an agamic complex with several ploidy levels which originated by autoploidy (Gates *et al*. 2004). Diploid cytotypes are sexual and self-incompatible while polyploids are self-fertile pseudogamous apomicts (Forbes and Burton 1961). Diploid cytotypes *P. notatum* var. *saurae* Parodi (Pensacola bahiagrass) (2n=2x=20) inhabits a relatively small area of Central Argentina, and tetraploids (2n=4x=40) (common bahiagrass) usually considered to be the typical form of the species in botanical terms, occupy a wide geographic area of South America (Daurelio *et al.* 2004). Sexual tetraploid plants are not found in nature, but some individuals were experimentally obtained from diploids (Forbes and Burton 1961, Quarin *et al.* 2003). The objective of this work was to perform a general survey of the frequencies and variation of cytosine methylation patterns at CCGG sequences in diploid and tetraploid cytotypes of *P. notatum,* and to determine the occurrence of methylation changes associated with autotetraploidization in the species.

Materials and methods

Diploid and tetraploid genotypes of *Paspalum notatum* Flüggé kept at the Instituto de Botánica del Nordeste, Corrientes, Argentina, were used. The diploid plants consisted of: 3-Cay and 6-Cay, collected within a natural population near Cayastá, Argentina; plant Q4175 derived from a natural population located approximately 15 km W of La Criolla, Argentina, and Tift9, one individual of cultivar Tifton 9, developed at the Coastal Plains Experimental Station, Georgia, USA. Tetraploids were: Q4117, an obligate apomictic individual from a natural population found in the state of Rio Grande do Sul, Brazil (Ortiz *et al.* 1997), N160 a facultative apomictic individual from a population located 25 km N of Pedro Juan Caballero, Paraguay (Espinoza *et al.* 2006) and two experimentally-generated tetraploid sexual plants Q4188 and Q4205 (Quarin *et al.* 2003). Moreover, the experimental series of a diploid plant C4-2x and its tetraploid counterpart C4-4x were used. C4-2x and C4-4x were regenerated from different sectors of colchicine treated callus, generated *in vitro* from young inflorescences of a natural diploid sexual genotype (Quarin *et al.* 2001).

Total genomic DNA was extracted from fresh leaves using the CTAB method according to Martínez *et al.* (2003). MSAP markers were generated following the protocol reported by Xu *et al.* (2000). The methylation-sensitive isoschizomers *Hpa*II and *Msp*I were employed. MSAP marker amplifications were carried out with 15 and 14 selective primer combinations for diploids and tetraploids plants, respectively. Amplification reactions were performed using the AFLP selective parameters as described by Vos *et al.* (1995). PCR products were denatured, electrophoresed and silver stained according to Espinoza *et al.* (2006). MSAP makers showing different methylation patterns were excised from the gels, eluted, re-amplified with the corresponding selective primer combinations and cloned in *pGEMTeasy* vector (*Promega*, Fitchburg, USA). At least 3 different clones of the same fragment were sequenced by *Macrogen* (Seoul, Korea). Consensus sequences were built with the *MegaAlign* module of *DNASTAR*, (Madison, USA). Annotation and similarity surveys were performed using *BLAST* tools available at *NCBI* (http://www.ncbi.nlm.nih.gov), *TIGR* (http://blast.jcvi.org/euk-blast) and *Gramene* (http://gramene.org/) web sites. Restriction analyses of cloned fragments were performed using the Cutter tool from *Justbio* - web page (www.justbio.com).

MSAP markers were scored as described by Cervera *et al.* (2002). Amplified fragments that differed in presence/absence for a given sample digested with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I were incorporated in a binary matrix of methylation-sensitive polymorphism (MSP). Alternatively, fragments that showed a monomorphic pattern for a given sample, but exhibited polymorphism between genotypes were used for creating a binary matrix of methylation-insensitive polymorphisms (MIP). Average proportions of MSP and MIP markers were compared using a *t*-test, at a significance level of $P < 0.05$. The Jaccard´s similarity coefficient (*J*) (Jaccard 1908) was estimated from each binary matrix. Dendrograms were created from each similarity matrix by the unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal 1973) using the *InfoStat* statistical software (www.infostat.com.ar). An estimate of the confidence limits for the grouping produced by each dendrogram was obtained by performing 5000 bootstrap resamplings with *WinBoot* (Yap and Nelson 1996). Correlation between MSP and MIP matrices were carried out by using the Mantel procedure with 10 000 permutations, at a statistical significance of $P < 0.05$ (Mantel 1967).

Results

MSAP analysis in diploid and tetraploid accessions of *P. notatum*: MSAP markers present in a DNA sample digested with both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I (pattern 11) were considered as originating from an unmethylated CCGG site. Markers displaying patterns 01 or 10 were regarded as arising from a methylated

CCGG sequence. Markers showing 00 patterns could be accounted for by either full cytosine methylation at a CCGG site or the absence of the target site (non-epigenetic difference) (Fig. 1). On average, 38.18 ± 7.32 and 31.88 ± 6.30 % of the CCGG sites were methylated in diploid and tetraploids groups, respectively (Table 1). Comparisons of the average proportion of methylated loci in diploid and tetraploid groups showed non-significant differences ($t = 1.47$; $P = 0.18$).

A methylation-sensitive polymorphism (MSP) matrix (that included 200 and 186 markers for diploids and tetraploids, respectively), and a methylation-insensitive polymorphism (MIP) matrix (that contained 283 and 332 markers for diploids and tetraploids, respectively)

Table 1. Levels of cytosine methylation at CCGG sites determined by MSAP markers in diploid and tetraploid genotypes of *P. notatum* (for detail see Material and methods).

Ploidy	Genotypes	Number of loci scored	Number of non-methylated loci	Number of methylated loci		
				total	*Eco*RI/*Hpa*II	*Eco*RI/*Mse*I
2x	3-Cay	363	214 (59.0 %)	149 (41.0 %)	12 (3.3 %)	137 (37.7 %)
	6-Cay	333	192 (57.7 %)	141 (42.3 %)	11 (3.3 %)	130 (39.0 %)
	Q4175	380	228 (60.0 %)	152 (40.0 %)	8 (2.1 %)	144 (37.9 %)
	Tifton 9	373	215 (57.6 %)	158 (42.4 %)	6 (1.6 %)	152 (40.8 %)
	C4-2x	887	663 (74.7 %)	224 (25.2 %)	53 (5.9 %)	171 (19.2 %)
4x	Q4117	377	255 (67.6 %)	122 (32.4 %)	12 (3.2 %)	110 (29.2 %)
	N160	278	192 (69.1 %)	86 (30.9 %)	6 (2.2 %)	80 (28.8 %)
	Q4188	408	280 (68.6 %)	128 (31.4 %)	7 (1.7 %)	121 (29.7 %)
	Q4205	255	150 (58.8 %)	105 (41.2 %)	9 (3.5 %)	96 (37.6 %)
	C4-4x*	881	674 (76.5 %)	207 (23.5 %)	41 (4.6 %)	166 (18.8 %)

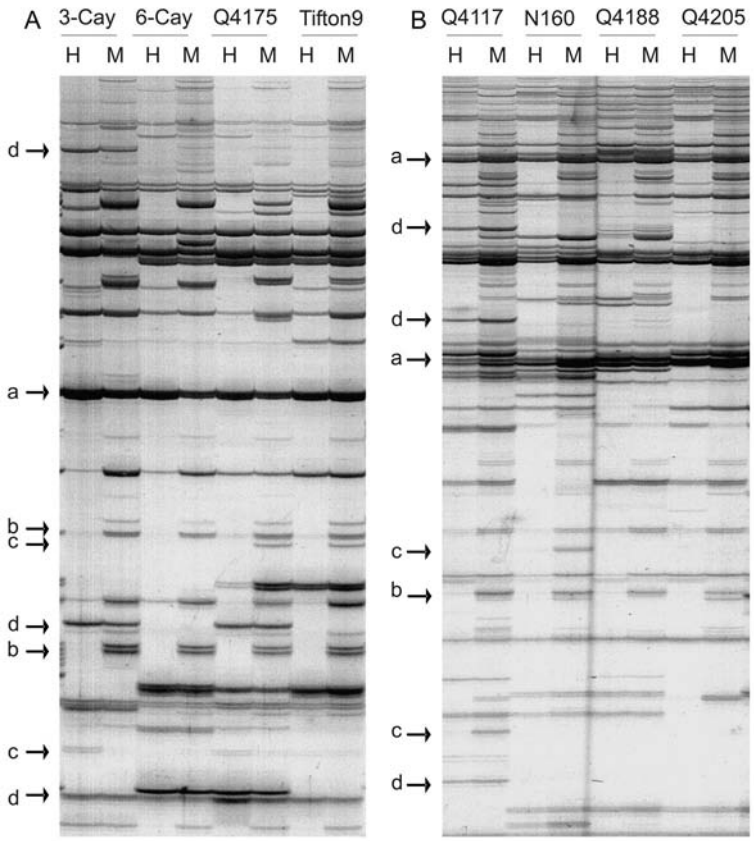

Fig. 1. MSAP markers in diploid (*A*) and tetraploid (*B*) genotypes of *P. notatum.* Markers were generated with primers *Eco*RI + AAG/*Hpa*II-*Msp*I + AGC (*A*) and *Eco*RI + AAT/*Hpa*II-*Msp*I + AGC (*B*). *Arrows* indicate markers corresponding to: a) unmethylated CCGG sites, b) and c) methylated sites (MSP) monomorphic or polymorphic between genotypes, respectively, d) methylation-insensitive polymorphism (MIP). H - sample digested with *Eco*RI/*Hpa*II and M - sample digested with *Eco*RI/*MspI*.

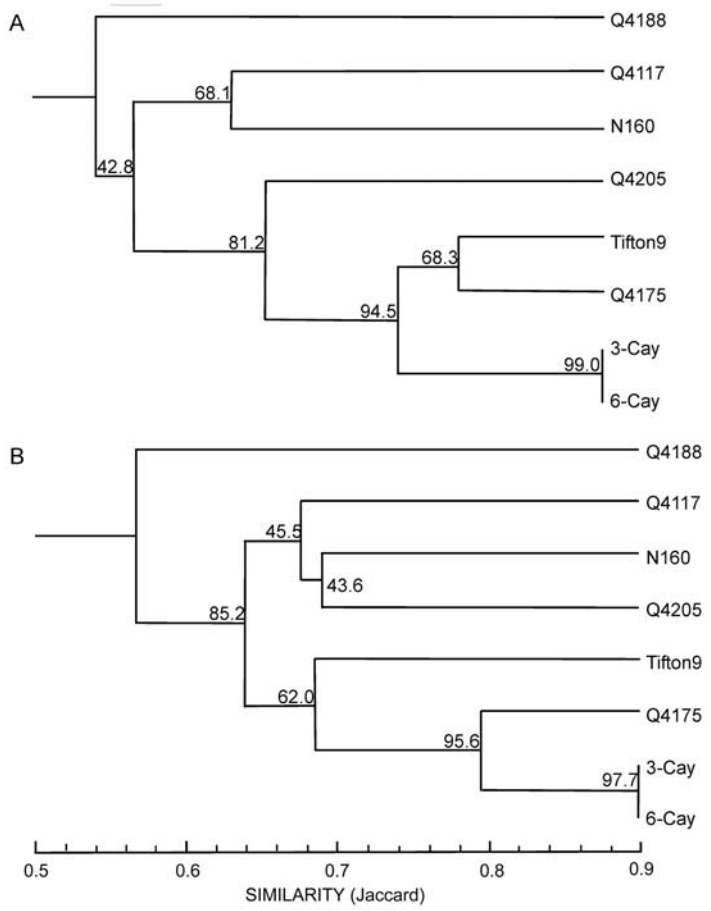

Fig. 2. Dendrogram of similarities constructed using the unweighted-pair group method with arithmetic averaging (UPGMA) based on MSP (*A*) and MIP (*B*) markers from diploid and tetraploid cytotypes of *P. notatum*. Bootstrap confidence intervals are included at the junctions of each cluster.

were created for generating epigenetic and non-epigenetic similarity estimates. On average, diploids showed an epigenetic similarity of J_{ep} = 0.76 ± 0.05. Genotypes 3-Cay and 6-Cay, both derived from the same natural populations, were the most similar (J_{ep} = 0.83). The lowest similarity value was found between 6-Cay and Tifton 9 (J_{ep} = 0.70). On the other hand, tetraploids showed an average epigenetic similarity of J_{ep} = 0.52 ± 0.04. Q4205 and Q4188, both derived from the same maternal plant showed an epigenetic similarity of J_{ep} = 0.54, while Q4205 and Q4117 showed a slightly higher value (J_{ep} = 0.57). The lowest epigenetic similarity was detected between Q4188 and N160 (J_{ep} = 0.46). The average epigenetic similarities of diploid and tetraploid groups were significantly different (t = 9.23; $P = 3.31 \times 10^{-6}$), indicating that tetraploids were more epigenetically variable than diploids. A similar analysis was carried out with data derived from methylation-insensitive polymorphisms (MIPs). Diploids showed an average non-epigenetic similarity of $J_{n\text{-}ep}$ = 0.71 ± 0.04. This value fits with the range of genetic similarity estimated for a group of diploid genotypes of the species (Daurelio *et al.* 2004). As expected, 3-Cay and 6-Cay were the more closely related ($J_{n\text{-}ep}$ = 0.77), followed by Q4175 ($J_{n\text{-}ep}$ = 0.71), all of which derive from central Argentina. The plant derived from the US cultivar Tifton 9 showed the lowest non-epigenetic similarity with 6-Cay ($J_{n\text{-}ep}$ = 0.67). The average of non-epigenetic similarity between tetraploids was $J_{n\text{-}ep}$ = 0.63 ± 0.06. This value was also in agreement with previous estimations of genetic similarities of tetraploid accessions of the species (Daurelio *et al.* 2004, Espinoza *et al.* 2006). The most similar genotypes were N160 and Q4117 ($J_{n\text{-}ep}$ = 0.69), followed by Q4117 and Q4205 ($J_{n\text{-}ep}$ = 0.67). The lowest non-epigenetic similarity of the group was obtained between Q4188 and N160 ($J_{n\text{-}ep}$ = 0.54). Comparison of the average non-epigenetic similarities of diploid and tetraploid groups showed significant differences (t = 3.18; P = 0.02), revealing that tetraploids were more diverse than diploids. Mantel´s test, performed with MSPs and MIPs matrices from both diploid and tetraploid groups, showed a positive correlation (*i.e.* $MSPs_{(2x)}$ *vs*. $MIPs_{(2x)}$ r = 0.882, P = 0.05 and $MSPs_{(4x)}$ *vs*. $MIPs_{(4x)}$ r = 0.948, P = 0.017), indicating that both epigenetic and non-epigenetic variation was related at both ploidy levels.

The analysis of a set of 159 MSP markers shared by both diploid and tetraploid genotypes showed 30 markers

(18.86 %) in which their methylation status varied with the change in the ploidy level. Twenty three markers, with identical methylation pattern in the 4 diploids, changed its methylation status in at least one of the tetraploids. On the other hand, 7 MSP markers with the same methylation pattern in the tetraploids showed a different pattern in at least one of the diploids. The dendrogram of epigenetic similarity with the set of common markers showed that all diploids and the tetraploid-sexual plant Q4205 clustered together. Bootstrap *P* value for this group was above 80 % (Fig. 2*A*). This outcome indicated that these plants shared a distinct epigenetic landscape despite the difference in the ploidy level. Another group was formed by the tetraploids Q4117 and N160 (with a bootstrap *P* value of 68.1 %), while Q4188 stayed apart of both groups (Fig. 2*A*). Clustering analysis with data derived from common MIP markers showed one group including the 4 diploids, (3-Cay, 6-Cay, Q4175 and Tifton 9) (bootstrap *P* value 62.0 %) and another group with three out of the four tetraploids (Q4205, Q4117 and N160) (bootstrap *P* value 45.5 %), while Q4188 remained a part of both groups (Fig. 2*B*).

Changes in methylation pattern of C4-2x and C4-4x: The analysis was carried out using 17 MSAP primer combinations that generated a total of 889 MSAP markers (229 MSPs and 660 MIPs). The majority of markers (73.90 %) showed a monomorphic pattern between plants. These fragments would be representing unmethylated CCGG sites at both C4-2x and C4-4x genotypes. 22.72 % of the markers showed the same

Table 2. Sequence analysis of MSAP markers showing different amplification patterns in *P. notatum*. Data from: [1]*Genebank* (http://www.ncbi.nlm.nih.gov/genbank), [2]*NCBI* (http://www.ncbi.nlm.nih.gov/) [3]*Blastx* (*NCBI*) (http://www.ncbi.nlm.nih.gov) or *TIGR* (www.jcvi.org) [4] *Gramene* (www.gramene.org).

Pattern	Name	Genotypes	Length [bp]	Accession No.[1]	*BLASTn*[2]	E-value	DNA or protein homolog[3]	Hits in rice[4]
A (1111)	A7	Q4188/Q4117	484	HM744740	gb|AC232774.1| *Solanum lycopersicum*	$5e^{-4}$	At1g73600 phosphoethanolamine N-methyltransferase 3 E = 0.024, P = 0.024	0
	A23	C4-2x/C4-4x	360	HM744741	gb|AC130732.2| *Oryza sativa*	$4e^{-3}$	none	115
B (0101)	B11	Q4188/Q4117	389	HM744742	none	-	none	0
	B26	C4-2x/C4-4x	315	HM744743	gb|AC134926.3| *Oryza sativa*	$6e^{-13}$	LOC_Os03g39210, retrotransposon protein, putative, unclassified E = 3e-14	123
C (1010)	C19	Q4188/Q4117	185	HM744744	ref|XM_002440910.1| *Sorghum bicolor*	$2e^{-30}$	none	0
D (1100)	D3	Q4188/Q4117	403	HM744745	emb|AM479609.1| *Vitis vinifera*	$4e^{-3}$	none	0
E (0011)	E8	Q4188/Q4117	382	HM744746	dbj|AP002902.2| *Oryza sativa*	$5e^{-21}$	ORSiTEMT00100011 gi|6979318|nt226750-227095 MITE, Tourist E = 5.4e-07, P = 5.4e-07	1013
	E32	C4-2x/C4-4x	270	HM744747	emb|AM432369.2| *Vitis vinifera*	$1e^{-2}$	At3g04910 68416.t00482 protein kinase family protein, E = 0.0079, P = 0.0078	7
F16 (0100)	F16	Q4188/Q4117	282	HM744748	gb|GQ353528.1| *Zea mays*	$4e^{-8}$	At4g02780 copalyl diphosphate synthase/ CPS E = 0.28, P = 0.25	121
	F18	Q4188/Q4117	332	HM744749	dbj|AP008212.1| *Oryza sativa*	$3e^{-05}$	none	11
G (0111)	G17	Q4188/Q4117	280	HM744750	ref|XM_002441819.1| *Sorghum bicolor*	$3e^{-89}$	LOC_Os12g06380, transposon protein, putative, unclassified, expressed, E = 9e-38	3
	G43	C4-2x/C4-4x	437	HM744751	emb|AM428644.2| *Vitis vinifera*	0.057	none	0
	G44	C4-2x/C4-4x	462	HM744752	gb|AC108498.2| *Oryza sativa*	$2e^{-13}$	LOC_Os05g12550.1 retrotransposon, putative, centromere-specific, E = 1.2e-18	35

methylation status at both the diploid and the tetraploid levels. These markers indicated methylated CCGG sites that remained unaltered after tetraploidization. A group of markers (1.12 %) showed variations that could be associated with genetic changes which occurred during polyploidization or alterations in the methylation status (full methylation/demethylation) of the target sites. Interestingly, 2.24 % of the markers showed differences (as a consequence of demethylation or hypermethylation) solely associated with the change in the ploidy level.

Sequence analysis of MSAP markers: Thirteen MSAP fragments representing different CCGG methylation patterns were cloned and sequenced (Table 2). Sequences representing non-methylated sites were first analysed to determine the homology of bands exhibiting the same migration rate in the gels. Alignment of consensus sequences of fragments A7 and A23 from both Q4188/Q4117 and C4-2x/C4-4x, respectively, showed more than 98 % of homology (data not shown) indicating that markers from the different genotypes corresponded to homologous sequences. MSAP fragments that showed the same methylation pattern in Q4188/Q4117 (B11 and C19) and C4-2x/C4-4x (B26), showed no homology to known sequences or match to a retrotransposon protein of rice, respectively. One of the fragments (E8) that showed a pattern related to a putative demethylation between Q4188/Q4117 was homologous to a MITE element of the Tourist class. Accordingly, 1103 alignments into the rice genome were detected. However, a similar type of marker from C4-2x/C4-4x (E32) showed homology with a protein kinase of *A. thaliana*. Thus, the same methylation pattern variation was detected in both repetitive and non-repetitive sequences. Interestingly, out of three markers (G17, G43 and G44) derived from a demethylation change at the tetraploid level (0111), two (G17 and G44) were homologous to transposon or retrotransposons proteins. Restriction analysis performed on all fragments listed in Table 2 showed that none of the fragments contained undigested internal CCGG sequences.

Discussion

The characterization of the epigenome of *P. notatum* at the diploid and tetraploid level could help to understand the mechanisms for adaptation, as well as, to determine the relation between polyploidy and the mode of reproduction. Our results indicate that the global methylation status remains relatively constant at both ploidies. However, the methylation pattern in tetraploids was more variable than in diploids. When comparing common markers, a group of MSP fragments that changed their methylation patterns at different ploidy was detected. Although, sequence variation could not be discarded, these markers may represent methylation modifications associated with the change in the ploidy level. Dendrograms based on epigenetic similarities showed that the tetraploid apomictic individuals clustered together, while the tetraploid sexual accessions were dispersed; particularly, Q4205 which grouped with the diploids (Fig. 2*A*). According to the origin of this plant, it may have retained the same epigenetic structure and pattern of the diploid plant from which it derived. On the other hand, Q4188 showed a different epigenetic makeup. This plant could have undergone a general epigenetic re-pattering as a consequence of receiving a male gamete from a natural apomictic genotype. MSP and MIP matrices showed positive correlations at both ploidy levels. This outcome could be as a consequence of stochastic epigenetic and non-epigenetic allelic modifications maintaining genome activity occurring in the formation of the polyploids.

Analyses carried out with C4-2x and C4-4x allowed us to determine the response of the *P. notatum* epigenome to an increment of the ploidy level based on an experimental system. While most markers maintained the same methylation status in both plants, about 2 % of them changed specifically as a consequence of polyploidization. Since in previous research works, variations and gene expression alterations associated with a change in the ploidy level were described in the same plant material (Martelotto *et al.* 2005, 2007), it could be assumed that polyploidization affects the *P. notatum* genome at both the genetic and epigentic levels. Sequence analysis of MSAP markers showed that methylation variation takes place at low copy genes, highly repetitive elements and non-coding sequences. Interestingly, several markers derived from a putative de-methylation in tetraploids corresponded to transposon or retrotrasposon sequences. Thus, repetitive elements could also play an important role in the increment of variability and adaptation of polyploid accessions existing in the agamic complex of *P. notatum*.

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